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EXTRACTION DE COMPOSANTS DE BIOMASSE LIGNOCELLULOSIQUE
OLEAGINEUSE EN MILIEU EAU ET CO₂ SUPERCRITIQUE
ET FONCTIONNALISATION ENZYMATIQUE

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Critical water and CO₂ mediated extraction of components from lignocellulosic and oleaginous biomass coupled to enzymatic functionalization.

This work addresses the integrated biorefining concept (extraction, fractionation, separation of compounds from biomass prior to further transformation) by developing discrete units with the ultimate objective of coupling them to enable a continuous flow configuration. Due to the complexity of solid, there is a need for a sustainable and environmentally friendly pre-treatment technology. Sub-critical water has been used as a solvent for extracting natural compounds in addition to hydrolysis. This work investigated the hydrolysis of carbohydrates (rice bran) and triacylglycerols (TAG; sunflower oil) chosen as models. The attribute of subcritical water (ion product and dielectric constant) in continuous flow reactors built for the purpose, allowed almost quantitative hydrolysis of hemicellulose and TAG. The effect of adding CO₂ and therefore carbonic acid was positive on the hydrolysis of hemicellulose. Further, free fatty acids were transformed to ethyl esters using lipase within continuous flow super critical CO₂ resulting in 95% yield. The hydrolysis and esterification reaction kinetics were studied. To address the complex interplay between multiple processing parameters response surface methodologies (RSM) were developed. Using the empirical data the models were successfully validated, therefore showing the utility of the RSM to assist process development. The important question of solubility of extractable in subcritical water was also addressed, through the development of a prediction method, validated with experimental data. In summary this work shows the possibility of applying the innovative Integrated Biorefining concept under continuous flow conditions -instead of the current application under batch conditions- for producing valuable compounds.

Keywords: Hydrolysis, extraction, esterification, response surface modeling, critical fluid

Extraction de composants de biomasse lignocellulosique et oléagineuse en milieu eau et CO₂ supercritique – Fonctionnalisation enzymatique

La thèse vise l'application du concept de bioraffinerie (extraction, fractionnement, séparation de composés à partir de biomasse avant transformation ultérieure), via le développement d'étapes de production destinées à être associées en un procédé continu. La complexité du solide nécessite une étape de prétraitement effectuée avec une technologie à faible impact environnemental et l'eau subcritique est déjà utilisée comme solvant d'extraction des produits naturels, en sus de leur hydrolyse. Ces travaux ont porté sur l'hydrolyse de polysaccharides (son de riz) et de triacylglycérols (TAG, huile de tournesol) choisis comme modèles. Les caractéristiques de l'eau subcritique (produit ionique, constante diélectrique) mise en œuvre en réacteurs à circulation construits dans ce but, ont permis l'hydrolyse quasi-totale de l'hémicellulose et des TAG. L'addition de CO₂ et donc d'acide carbonique a eu un effet positif sur l'hydrolyse de l'hémicellulose. Les acides gras libres résultant de l'hydrolyse ont été estérifiés en ester éthyliques en présence d'une lipase en réacteur continu en milieu CO₂ supercritique, avec un taux de synthèse de 95%. Les cinétiques des réactions ci-dessus d'hydrolyse et d'estérification ont été étudiées. La complexité des interactions entre les nombreux paramètres mis en jeu ont conduit à appliquer des méthodes de plans d'expérience. Ces méthodes ont été validées avec succès avec les données expérimentales, montrant ainsi leur utilité dans le développement de procédés. La question importante de la solubilité des extractibles dans l'eau subcritique a été traitée et une méthode de prédiction mise au point et validée avec succès avec les données expérimentales. En conclusion, ce travail montre la possibilité d'appliquer le concept novateur de la Bioraffinerie Intégrée en réacteur continu avec des fluides sub- ou supercritique, contrairement à leur mise en œuvre actuelle en réacteur fermé, pour la production de composés commercialisables.

Mots-clés : Hydrolyse, extraction, estérification, plan d'expérience, fluide critique

Publication output from the Thesis

(Main publications in Appendix D)

Internationally referred Journals

- 1) Baig M.N., Santos R.C.D, Zetzi C., King J., Pioch D., Bowra S., Evaluation and modeling the utility of SCCO₂ to support efficient Lipase mediated esterification; *Enzyme and Microbial Technology* 2011;49(4), 420-426
- 2) Baig M.N., Santos R.C.D, Zetzi C., King J., Pioch D., Bowra S., Evaluation and modeling of continuous flow sub-critical water hydrolysis of biomass derived components; lipids and carbohydrates; *Chemical Engineering Research and Design* (Submitted 2012)
- 3) Roquea, R.M.N., Baig, M.N. Leek, G.A. Bowra, S. Santos, R.C.D. Study on sub-critical water mediated hydrolysis of *Miscanthus* a lignocellulosic biomass; *Resources, Conservation and Recycling* (In Press)
- 4) Alenezi R., Baig M.N., Wang J., Santos R.C.D., Leeke G.A., Continuous Flow Hydrolysis of Sunflower Oil for Biodiesel. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, 2010. 32(5): p. 460 - 468.

International conference communications

- 1) Baig, M.N, Santos, R., King, J.W., Bowra, S., and Pioch, D. Optimizing the potential of critical fluids to support a sustainable and integrated bio-refining platform with respect to oleochemical production. *Proceedings of the 10th International Symposium on Supercritical Fluids (ISSF-2012)*, San Francisco, CA, May 13-16, 2012. Proceeding P-1207, pp. 2052-2059.
- 2) Baig, M.N, Santos, R., King, J.W., Bowra, S., Zetzi, C., Brunner, G. and Pioch, D The potential of critical fluids to support sustainable integrated biorefining with a focus on oleochemistry 5th Workshop on Fats and Oils as Renewable Feedstock for the Chemical Industry Karlsruhe, Germany, March 18 – 20, 2012
- 3) Baig M.N., Santos R.C.D, University of Birmingham (UK), Bowra S., Phytatec Ltd.(UK); Pioch D., University of Montpellier (France), One step extraction of vegetable oil and lipase mediated synthesis of commodity oleochemicals under critical fluid conditions: case of fatty acid ethyl esters 102nd AOCS Annual Meeting & Expo (Cincinnati Ohio, USA, 2011).
- 4) Baig M.N., Santos R.C.D, University of Birmingham (UK), Bowra S., Phytatec Ltd.(UK); Pioch D., University of Montpellier (France), Modelling of critical fluids process for adding value and functionality to sunflower oil; proceedings of 4th Workshop on Fats and Oils as Renewable Feedstock for the Chemical Industry (Karlsruhe, Germany, 2011).
- 5) Roque R., Baig M.N., Leeke G.A., Santos R.C.D., Bowra S. University of Birmingham (UK); Sub-critical water mediate hydrolysis and fractionation of lignocellulosic Biomass (*Miscanthus x giganteus*); Bioten; (Birmingham, United Kingdom 2010).
- 6) Baig M. N., Bowra S., Santos R.C.D; University of Birmingham (UK); Green process for the continuous production of Hydroxymethylfurfural (HMF) from biomass; Bioten; (Birmingham, United Kingdom 2010).
- 7) Baig M.N., Alenezi R., Leeke G.-A., Santos R.-C.-D, University of Birmingham (UK), Zetzi C., TUHH (Germany), King J., University of Arkansas (USA), Pioch D., University of Montpellier (France) and Bowra S., Phytatec Ltd.(UK), Critical fluids as process environment for adding value and functionality to sunflower oil; a model system for biorefining, proceedings of 11th European meeting on supercritical fluids – Key note (Barcelona, Spain, 2008).
- 8) R. Alenezi, M.N.Baig, G.A. Leeke, R.C.D Santos, University of Birmingham (UK), Continuous flow hydrolysis of sunflower oil using sub-critical water, proceedings of 11th European meeting on supercritical fluids (Barcelona, Spain, 2008).
- 9)) R. Alenezi, M.N.Baig, R.C.D Santos, G.A. Leeke, Catalyst free production of biodiesel, Universitas 21, International conference on Energy Technology and Policy (Birmingham, UK 2008)

ABBREVIATIONS

CNG	Compressed natural gas
CCD	Central composite design
DAG	Diacylglycerol
DP	Degree of polymerization
FAEE	Fatty acids ethyl esters
FAME	Fatty acids methyl esters
FFA	Free fatty acids
FID	Flame ionization detector
G	Glycerol
GC	Gas chromatography
HPLC	High performance liquid chromatography
LPG	Liquid petroleum gas
MAG	Monoacylglycerol
MS	Mass spectroscopy
O/C ratio	Oxygen/carbon ratio
PFR	Plug flow reactor
PHEV	Plug in hybrid electric vehicle
Re	Reynolds number
RTD	Residence time distribution
RTFO	Renewable transport fuels obligation
S/C ratio	steam/carbon ratio
SCCO ₂	Super critical carbon dioxide
SCW	Super critical water
SCF	Super critical fluid
TAG	Triacylglycerol
TPO	Temperature programmed oxidation

Greek symbols

ε	watts/kg	Energy dissipation rate
Φ	J/kg	Energy dissipation
ρ	kg/m ³	Density

ρ_c	kg/m^3	Density of continuous phase
μ	kg/m.s	Viscosity
ν	m^2/s	Kinematic viscosity
σ	N/m	Surface tension
τ	Pa	Shear stress

Nomenclature

A_o	Pre-exponential factor
D	Diameter of tube, m
E_a	Activation energy
k	Reaction rate constant, $(\text{min}^{-1}[\text{mol/mol of oil}]^{-1})$
L	Length, m
P	Pressure, MPa
R	Gas constant, $8.314 \text{ J}/(\text{mol.K})$
Re	Reynolds number
t	Reaction time, min or s
T	Temperature, °C or K
V	Volume, mL

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RESUME EN FRANÇAIS

Introduction - Objectif de la thèse

L'objectif de la thèse est d'étudier plusieurs étapes clés d'une bioraffinerie qui traiterait la biomasse lignocellulosique et oléagineuse, dans le but de tester la faisabilité industrielle. Ces résultats intéressent la société Phytatec UK Ltd, laquelle a financé les travaux et la bourse d'étude du doctorant (alors salarié de l'entreprise). La biomasse choisie, de type oléagineux, correspond à une matière première de composition chimique plus complexe que celle des substrats actuellement utilisés ou tout au moins envisagés pour la production de biocarburant de deuxième génération (éthanol). Cette complexité doit conduire à la production de plusieurs fractions aux propriétés bien différentes : extractibles hydrosolubles, extractibles de faible polarité (huile), composants de la matrice lignocellulosique, mais aussi à des dérivés de ces derniers, intégrant alors déconstruction, fractionnement, et fonctionnalisation des extraits. La société Phytatec, déjà engagée dans les technologies à faible impact environnemental, souhaitait tester le potentiel des fluides à l'état critique, sur la base de leurs avantages promus via de nombreux travaux scientifiques. Il s'est agi de rechercher des conditions d'applications compatibles avec les contraintes de la production industrielle.

Le mémoire est organisé en 12 chapitres. La synthèse bibliographique (Partie A) est elle-même subdivisée en cinq chapitres, faisant le point dans les grands domaines à l'intersection desquels se situe la thèse : huiles et lipochimie (Chapitre 1); polymères naturels et traitement de la matière lignocellulosique (Chapitre 2); enzymes et usages industriels (Chapitre 3); applications industrielles des fluides supercritiques (Chapitre 4) ; bioraffinerie (Chapitre 5).

La Partie B rassemble la description des matériels et des méthodes utilisés : protocoles analytiques (Chapitre 6), méthodes de calcul, cinétique, optimisation, modélisation (Chapitre 7), dispositifs et protocoles d'extraction en batch et continu (Chapitre 8).

Les autres chapitres exposent les résultats en Partie C. Le Chapitre 9 traite de l'hydrolyse non catalysée du son de riz (déshuile) comme modèle de biomasse lignocellulosique avec l'eau subcritique, et l'hydrolyse de l'huile est réalisée au Chapitre 10.

L'estérification des acides gras libres avec l'éthanol par voie enzymatique fait l'objet du Chapitre 11, comme exemple de fonctionnalisation des premiers produits vers des produits « de spécialité ». Enfin le Chapitre 12 traite de la solubilité des extractibles dans l'eau subcritique.

Revue bibliographique

L'étude bibliographique, étendue par nécessité, couvre les grands domaines à l'intersection desquels se situe la thèse : connaissance et transformation de la biomasse oléagineuse et lignocellulosique, procédés de déconstruction, extraction, fonctionnalisation à faible impact environnemental. C'est justement dans cette démarche « d'intégration » que le sujet de thèse trouve sa justification.

La matière première. La biomasse est une matrice d'une très grande complexité, au niveau moléculaire et cellulaire, contenant une grande variété de polymères, amidon, polysaccharides, mais surtout lignine, hemicellulose et cellulose. La cellulose, polymère linéaire de glucose (degré de polymérisation ~ 10.000) est la matière de base formant les fibres. L'hémicellulose, polymère ramifié de glucose et de xylose, de structure amorphe remplit les interstices entre chaînes de cellulose. La lignine, composée de monomères phénoliques, plus pauvre que les précédentes en atomes d'oxygène, forme un réseau polymérique réticulé, servant de liant entre les fibres cellulosiques. La cellulose est le polymère naturel le plus répandu sur Terre, et la biomasse lignocellulosique, vu son faible coût, est très compétitive au plan économique. Evidemment de nombreuses utilisations de ces composés naturels existent, depuis les fibres textiles de cellulose, jusqu'à la lignine servant de colle, en passant par la pâte à papier. Cependant l'utilisation de la biomasse lignocellulosique en chimie industrielle est limitée par les procédés de dépolymérisation, étape indispensable pour disposer des briques de base –monomères, oligomères- et alimenter le secteur chimique.

Ce haut degré de structuration constitue un frein à l'action des agents chimiques et a fortiori enzymatiques, susceptibles de dépolymériser. Pour cette raison une large palette de traitements, en fait appelés « prétraitements », est proposée, de nature physique ou mécanique incluant l'explosion par dépressurisation rapide, chimique (acides, bases, agents oxydants), ou biologique.

Les extractibles de la biomasse sont des composés de faible poids moléculaire, métabolites destinés par exemple à défendre la plante contre les agressions, ou des composés de stockage d'énergie tels les huiles. Ces dernières, rencontrées dans des cellules spécialisées des graines sont d'ailleurs à l'origine des cultures oléagineuses. Une dizaine environ couvre les besoins de l'alimentation humaine, mais une partie de la production ($\sim 10\%$) est de plus en plus utilisée par le secteur lipochimique pour la production de savons et de composés lipochimiques de base (acides gras, esters méthyliques, alcools, amines, glycérol), destinés à de nombreuses applications

(lubrifiants, détergents, émulsifiants), récemment comme carburants pour moteurs diesel. L'huile de palme est la première production oléagineuse à l'échelle mondiale, devant le soja, et la principale matière première pour la lipochimie, laquelle est donc principalement alimentée par une culture tropicale.

Les huiles sont composées de triacylglycérols, esters d'acides carboxyliques à longue chaîne hydrocarbonée et de glycérol, et par définition elles ne sont pas hydrosolubles. Leur extraction fait donc appel à un solvant organique (hexane) lorsque la simple pression ne suffit pas. En sus de l'hydrogénation des doubles liaisons $C=C$, les réactions au niveau de la fonction carboxylique, hydrolyse et alcoololyse, sont la principale voie de fonctionnalisation des triacylglycérols, essentiellement par catalyse chimique, mais aussi par catalyse enzymatique.

Biocatalyse. Les enzymes sont des protéines jouant le rôle de catalyseurs dans le monde vivant. Le site actif est constitué par des acides aminés formant une triade, dont la proximité spatiale résulte de la structure tertiaire de la protéine. Les sites sont spécifiques d'une fonction ou d'une réaction donnée, ce qui fait des enzymes des catalyseurs extrêmement sélectifs. Les lipases, appartenant au groupe des hydrolases-liases, dédiées à la fonction carboxylique des acides gras sont d'un intérêt particulier pour la thèse.

Ces catalyseurs sont produits à l'échelle industrielle par culture de microorganismes et purification-séparation des cellules ou débris cellulaires. Les enzymes extracellulaires (attachées à l'extérieur des parois) sont plus faciles à isoler. Les enzymes industrielles sont principalement destinées aux applications alimentaires (synthèse de triacylglycérols comme substituts de beurre de cacao), mais pas seulement (lessive, industrie du cuir). Outre leur sélectivité, leur avantage réside dans les conditions de travail proches de la température ambiante, compatibles avec le traitement de composés thermodégradables, permettant des économies d'énergie, et dans la limitation des rejets chimiques polluants.

Fluides supercritiques. Ils sont souvent mentionnés pour le traitement des produits naturels. Il s'agit de gaz ou de liquides dans les conditions normales, utilisés dans des conditions de température et de pression au-delà du point critique (T_c , P_c). Ils présentent alors une densité et un pouvoir solvant proches de ceux des liquides, tout en conservant une viscosité et une diffusivité plus proches de celles des gaz. De plus les fluides supercritiques n'ont pas de tension interfaciale. Cet ensemble de propriétés leur permet de pénétrer plus facilement à l'intérieur des matrices végétales, facilitant ainsi le transport de matière et l'extraction. De plus la solubilité est très sensible aux conditions de température et de pression, ce qui permet d'obtenir des sélectivités

élevées et de fractionner au sein d'une même classe de composés. Certaines de ces propriétés restent intéressantes au voisinage –mais aussi en–delà– du point critique, correspondant donc à des gaz ou liquides comprimés. Ces conditions, globalement dénommées « subcritiques », présentent l'avantage de nécessiter moins d'énergie notamment pour la compression.

Au plan des applications l'eau (P_c 22MPa, T_c 374°C) est souvent proposée dans des conditions subcritiques, plus faciles d'accès que l'état supercritique, ainsi que le CO₂, sub- ou supercritique (P_c 7.3 MPa, T_c 31.1°C) qui est déjà mis en œuvre à l'échelle industrielle (décaféinisation, huiles essentielles). Dans la presque totalité des cas les procédés sont de type discontinu, ce qui est un frein à la viabilité économique. Le fluide est décomprimé pour permettre la récupération de l'extrait par insolubilisation, puis recyclé.

La bioraffinerie. Sous l'acception qui intéresse la thèse, c'est un concept analogue à celui de la raffinerie pétrolière, du fait que l'ensemble des composants de la biomasse qui alimente l'usine est censé trouver un usage, contribuant ainsi à maximiser la valeur des produits et à réduire les coûts de production. Il s'agit plus d'un concept que d'une réalité industrielle, dans la mesure où les usines en production ou en construction ont pour objectif de produire principalement un seul composé, l'éthanol principalement, utilisé comme carburant. Cette simplification à l'extrême du concept de bioraffinerie qui reste innovante dans la mesure où l'ensemble de la biomasse de départ est censée être fermentée en alcool, se différenciant ainsi de la distillerie classique, est justifiée par les nombreuses incertitudes qui pèsent sur la disponibilité et sur les performances des technologies indispensables, souvent très innovantes, par le montant élevé des investissements, par les incertitudes sur les marchés s'il s'agit de produits nouveaux -ce qui n'est pas le cas de la production d'un composé déjà sur le marché, tel l'éthanol, ou l'acide lactique destiné à la polymérisation.

En conclusion. Il ressort de cette synthèse bibliographique que le degré de structuration élevé et la complexité de composition chimique de la matrice végétale d'une part, et d'autre part la disponibilité de procédés bien adaptés aux différentes contraintes d'efficacité (échelle, sélectivité), à la production de plusieurs fractions, à la minimisation de l'impact sur l'environnement, sont des freins au développement de ces nouvelles filières agro-industrielles.

La présente thèse a pour objectif de contribuer à explorer plusieurs options au plan des procédés.

Méthodes et dispositifs expérimentaux

Au plan expérimental, en sus des méthodes d'analyse des produits, il convient de mentionner :

- la mise en œuvre de plusieurs méthodes de modélisation au plan cinétique et d'optimisation des conditions expérimentales, de calcul de la solubilité de produits naturels dans l'eau subcritique,
- la conception et la construction de trois unités travaillant en continu et sous pression, adaptées aux spécificités des réactifs et des produits (sécurité, corrosion des alliages) et des réactions mises en œuvre jusqu'à 350°C et 30 MPa, suivant le cas, équipés des dispositifs de régulation de température et de pression et de dépressurisation-échantillonnage des produits :
 - réacteur d'hydrolyse en continu alimenté avec une dispersion aqueuse de son de riz, et doté d'une deuxième pompe d'alimentation en CO₂,
 - réacteur d'hydrolyse en continu d'huile de son de riz alimenté à co-courant via deux pompes doseuses (eau, huile),
 - réacteur d'estérification en continu à lit catalytique fixe (lipase supportée dispersée dans des billes de verre), alimenté par trois pompes doseuses (mélange préalable dynamique des flux d'éthanol et d'acides gras libres), avant l'alimentation en CO₂.

Orientation de l'étude

Parmi les gisements de biomasse pour la bioraffinerie, cultures dédiées ou coproduits de filières alimentaires ou forestières existantes, la deuxième option a été privilégiée. Il a été décidé de travailler sur le son de riz qui est un sous-produit de l'une des principales filières alimentaires au plan mondial. Le son de riz est à la fois une biomasse lignocellulosique et oléagineuse, et constitue de ce point de vue un modèle intéressant.

Cependant l'extraction de l'huile n'a pas été vue comme une étape prioritaire dans le cadre de la thèse, forcément limité. En conséquence les substrats utilisés sont le son de riz déshuilé d'une part (bagasse après extraction de l'huile par l'hexane) et d'autre part l'huile elle-même, en tant que produit intermédiaire, et dont les conditions d'extraction y-compris par SCCO₂ sont connues.

La transition de la pétrochimie -basée sur des hydrocarbures seulement comme substrat de départ- vers la chimie des composés déjà oxygénés qui composent l'essentiel de la biomasse, présente plusieurs verrous, notamment la réalisation de transformations chimiques dans des conditions viables aux plans économique et environnemental, tout en évitant la dégradation des produits d'intérêt. Parmi ces verrous la réaction de dépolymérisation de la matrice végétale a été choisie.

Les huiles quant à elles sont une source de chaînes hydrocarbonées déjà fonctionnalisées, offrant un accès facile aux acides carboxyliques et à leurs dérivés (esters, alcools, amines, dimères ...).

La nécessité de disposer de procédés fonctionnant en continu, qui est apparue à la suite de l'étude bibliographique a conduit à tester les réactions ci-dessus avec l'eau subcritique, en présence ou non de CO₂ afin d'utiliser au mieux le faible choix d'options « chimiques », ayant volontairement écarté les catalyseurs en phase homogène, acides ou basiques (corrosion, effluents) et d'autres options dont l'intérêt est incertain au plan économique.

Après avoir obtenu les acides gras libres par hydrolyse d'une huile, leur transformation en esters éthyliques a aussi été testée, comme exemple de fonctionnalisation des produits intermédiaires; les esters éthyliques étant –encore– des spécialités chimiques et non des composés lipochimiques de base, mais ils sont vus aussi comme un biodiesel potentiel.

Hydrolyse du son de riz délipidé en réacteur continu par l'eau subcritique avec ou sans CO₂

Une unité de laboratoire a été construite, permettant de travailler jusqu'à 250°C et 20 MPa, dans laquelle le réacteur tubulaire de 50 mL est alimenté avec une dispersion de son de riz broyé, à 1% pondéral dans de l'eau distillée. La teneur en carbone de l'hydrolysate a été mesurée après séparation du solide, afin d'accéder au taux d'hydrolyse de la biomasse, par comparaison à la teneur initiale de la suspension. Le domaine de variation des deux paramètres étudiés est de 150 à 210°C et jusqu'à 60 minutes de temps de résidence. Le taux d'hydrolyse par exemple à 60 minutes et 20 MPa, augmente de 5 à 35% lorsque la température passe de 150 à 220°C. Ce résultat est à rapprocher de ceux de la littérature (même taux d'hydrolyse de la bagasse de canne à sucre à 210°C après 20 minutes en réacteur fermé). L'allure générale des cinétiques montre deux périodes, d'abord une évolution quasi linéaire et assez rapide, puis un infléchissement, qui se poursuit par une évolution beaucoup plus lente.

Cette allure laisse présager de l'hydrolyse de deux fractions de nature chimique différente ou situées dans des environnements différents (accessibilité). Si cette inflexion est peu marquée à 150°C, il n'en est pas de même à 180 et 200°C. Elle intervient vers 20-25 minutes, mais le taux de conversion « seuil » est fonction de la température, 5 et 20% pour 150 et 220°C respectivement.

Pour ce qui est de la composition des produits, la cinétique montre clairement la formation de xylose comme principal premier produit (25 % des produits en poids) et de glucose comme produit minoritaire. Ces deux composés sont aussi transformés in situ en acide formique, furfural et 5-hydroxyméthylfurfural, l'acide formique devenant le produit principal après 50 minutes. Ces résultats recoupent en partie ceux de Rogalinski et al (2008), qui ont obtenu 10% de furfural à

partir de paille de riz, en 10 minutes à 120°C. L'étude a permis d'accéder à l'énergie d'activation de la réaction globale de l'hydrolyse (46 KJ/mole).

L'addition de CO₂ exerce un effet positif sur le taux de conversion qui tend à se réduire au-dessus de 200°C. Cet effet positif s'explique par l'acidification du milieu, du fait de la formation d'acide carbonique participant à la catalyse de la réaction, alors que dans le cas de l'eau seule, celle-ci est réalisée grâce à la seule présence des ions hydroxyde et des protons résultant de la dissociation de l'eau subcritique (produit ionique plus élevé par rapport à l'eau liquide). De plus il semble logique d'invoquer l'autocatalyse dès qu'apparaît l'acide formique, produit de dégradation poussée.

Si la sélectivité vis-à-vis des différents produits et coproduits d'hydrolyse ne peut pas être orientée pour atteindre 100%, ce travail en réacteur continu laisse voir la possibilité de moduler les conditions de réaction en fonction des produits recherchés, et montre la faisabilité de l'hydrolyse en continu, vers la « liquéfaction » de l'hémicellulose, bien que les conditions de réaction n'aient pas été optimisées, notamment le rapport charge solide/eau afin d'éviter de chauffer un trop grand volume d'eau à 200°C.

Hydrolyse de l'huile de tournesol en réacteur continu en milieu eau subcritique

L'hydrolyse de l'huile de tournesol en acides gras et en glycérol est une étape de base de l'industrie lipochimique et donc de l'exploitation de la filière oléagineuse. Actuellement cette étape (fat-splitting) est réalisée avec de l'eau à 250°C et 5 MPa, par injection de vapeur. Au cours de ce travail nous avons étudié l'impact de conditions plus proches de l'état critique (270-350°C, 20 MPa, eau/alcool 1/1 – ¼ v/v). Les résultats montrent que l'hydrolyse des acylglycérols est complète après 8 à 15 minutes, réduisant donc le temps d'un facteur dix par comparaison aux procédés actuels, et sans ajout de catalyseur ni de CO₂. De plus ces conditions permettent de réaliser une seconde transformation des acides gras en augmentant la température et le temps de résidence. Bien que ces seconds produits n'aient pas été analysés, la présence de C18:2 en tant que principal acide gras laisse déduire d'après la littérature qu'ils pourraient être des acides dimères et des polymères, déjà commercialisés comme composants de colles et de peintures. D'un point de vue appliqué, ces conditions fournissent donc un procédé permettant l'accès à des produits de base (acides gras, glycérol), ou à des composés de spécialité ayant une valeur supérieure.

Au plan des réactions chimiques, il a été observé que le rendement en produits primaires (acides gras) dépend de la température, du temps de réaction, du rapport eau/huile et de la densité de l'eau. Un rendement et une sélectivité élevés en acides gras nécessite une température modérée

(inférieure ou égale à 300°C) et un donc un temps de séjour plus long, afin d'éviter la formation des seconds produits. L'effet catalytique mentionné dans littérature a été noté et mesuré via le pH du produit brut d'hydrolyse en sortie de réacteur: conversion élevée de l'huile et pH faible sont liés (toutefois l'identification des acides dissociés responsables de ce phénomène ne faisait pas partie des objectifs de la thèse). De plus les propriétés particulières de l'eau subcritique (produit ionique élevé, stabilisation accrue des intermédiaires de réaction ioniques) sont de nature à faciliter l'hydrolyse. Enfin, ces réactions d'hydrolyse présentent un temps d'induction et à partir de résultats non inclus au mémoire (Baig et al., Submitted 2012) nous avons observé la disparition de cette période d'induction dans le cas d'huiles dotées d'une acidité élevée (son de riz, suif), effet positif de l'acidité initiale aussi observé par d'autres équipes. Cette période d'induction est expliquée par un phénomène d'autocatalyse attribué à l'accélération de la réaction par les acides gras libres néoformés, lequel paraît alors prépondérant en l'absence de catalyseur ajouté.

Un tel procédé en milieu eau subcritique serait donc très souple, acceptant des substrats de faible cout (huiles non raffinées ayant même une acidité élevée). Ces résultats donnent donc les bases pour une optimisation ultérieure dans les meilleures conditions techniques et économiques, de ce procédé d'hydrolyse en continu, basé seulement sur les propriétés de l'eau subcritique et pouvant conduire à plusieurs produits au choix, en une seule étape.

Synthèse d'ester éthylique d'acide gras en milieu CO₂ supercritique catalysée par une lipase

Le dernier exemple étudié au cours de la thèse concerne une étape de fonctionnalisation d'un produit primaire de la bioraffinerie. L'acide oléique qui pourrait résulter de l'hydrolyse ci-avant a été estérifié en ester éthylique en présence d'une lipase (Lipozyme TL IM, Novozyme) en réacteur continu en milieu éthanol plus CO₂ supercritique, avec un taux de synthèse de ~95%. L'influence des paramètres réactionnels et la cinétique de la réaction ont été étudiées. La modélisation à l'aide d'un plan d'expérience a permis d'obtenir les conditions optimales et ce résultat a été validé par l'expérience aux approximations près introduites dans le modèle (95 au lieu de 100%). Le faible impact de la pression sur la stabilité de l'enzyme est un point important, tout comme le fonctionnement en réacteur continu qui accélère la réaction (95% de conversion pour 60 min de temps de séjour, 60°C, 20 MPa, éthanol/acide gras 2/1, 11% p/v d'enzyme dans le réacteur), par comparaison au procédé en réacteur fermé (55% en 6 h, avec 10%p d'enzyme par rapport à l'acide, en présence de CO₂). Toujours dans ces mêmes conditions en batch mais sans CO₂, la conversion est limitée à 38%, l'équilibre réactionnel étant visiblement encore plus perturbé par l'eau néoformée, coproduit de l'estérification. Ici aussi ces résultats montrent l'effet bénéfique du

CO₂. Une meilleure connaissance du milieu réactionnel permettrait d'améliorer le procédé en continu, pour déplacer l'équilibre vers 100% de synthèse.

Modélisation de la solubilité des extractibles dans l'eau subcritique

La question importante de la solubilité des extractibles et des produits de réaction dans l'eau subcritique, notée au cours de la mise au point des conditions expérimentales, a été traitée et une méthode de prédiction applicable jusqu'à 400°C, a été mise au point et validée avec succès avec les données expérimentales. Elle accepte divers milieux susceptibles d'être mis en œuvre dans le cadre de la bioraffinerie, notamment eau et méthanol subcritiques. Cette méthode fournira donc les informations nécessaires à la mise en œuvre des procédés et à leur optimisation ; la solubilité des substrats et des produits gouverne ces procédés et les différences de solubilité sont mises à profit pour déplacer les équilibres ou séparer sélectivement les produits des milieux réactionnels.

Conclusion générale

La transition de la pétrochimie, basée sur des hydrocarbures comme substrat de départ, vers la chimie de dépolymérisation-fonctionnalisation à partir de composés déjà oxygénés qui composent l'essentiel de la biomasse, est à la fois un défi mais aussi une source d'opportunités pour l'industrie chimique. Par exemple l'utilisation d'hydrates de carbone élimine le recours systématique aux réactions d'oxydation qui sont au centre de la pétrochimie (alcools, acides, esters, cétones), avec accès à des sites chiraux si nécessaire. Les huiles quant à elles sont une source de chaînes hydrocarbonées déjà fonctionnalisées, offrant un accès facile aux acides carboxyliques, esters et amines, contrairement aux longues synthèses qui caractérisent souvent la pétrochimie.

Les trois réactions testées à titre d'exemple au cours de ce travail –hydrolyse de l'hémicellulose ; hydrolyse des triacylglycérols ; estérification des acides gras libres en esters éthyliques- ont permis d'évaluer les avantages de l'eau subcritique et du CO₂ supercritique au plans chimiques et physicochimiques pour optimiser ces réactions, en jouant sur les propriétés différentes de ces fluides dans un état proche du point critique (eau subcritique et CO₂ supercritique), sans utilisation de catalyseurs autres qu'une lipase pour la troisième étape et de CO₂ pour la première. Un effort a été fait en modélisation de ces systèmes réactionnels complexes et les modèles ont été validés de façon acceptable par les résultats expérimentaux.

Cependant l'étude de ces réactions a été limitée par le déficit de connaissances aux plans physique et physicochimique. L'optimisation de ces étapes en milieu fluides critiques nécessite la prise en compte de la solubilité des réactifs (produits naturels) et des produits, dans ces milieux complexes, et son évolution en fonction du taux de conversion et en sortie de réacteur continu. Cette connaissance est essentielle pour le développement des procédés, en milieu eau subcritique pour ce qui est du présent travail, et la méthode développée permet de calculer la solubilité de nombreuses classes de produits intéressant la bioraffinerie.

La complexité des interactions entre les nombreux paramètres mis en jeu a conduit à appliquer des méthodes d'optimisation basées sur des plans d'expérience. Ces méthodes ont été validées avec les données expérimentales, montrant ainsi leur utilité dans le développement de procédés. Elles ont aussi permis d'approcher les interactions et les synergies entre ces paramètres.

Au cours de ce travail, priorité a été donnée d'une part à la construction de dispositifs expérimentaux adaptés aux différentes contraintes imposées par les réactifs et les réactions visées, et par l'objectif essentiel de parvenir à mettre le tout en œuvre en réacteur continu, et d'autre part à la recherche de conditions expérimentales favorables, à défaut d'une optimisation complète.

En conséquence la suite de ce travail mérite un effort particulier pour l'analyse chimique des produits de réaction, ainsi qu'une attention particulière aux nombreuses réactions possibles et aux mécanismes réactionnels associés, l'ensemble de ces connaissances chimiques approfondies permettra alors d'améliorer l'efficacité des différentes étapes du procédé en gestation, rendements et sélectivités. Par exemple il a été noté que le ratio eau/huile pouvait avoir des effets contraires en fonction de la pression, sur le déroulement de l'hydrolyse des acylglycérols. L'effet de la pression est d'autant plus marqué que le ratio est plus faible, et il semble dépendre du taux d'hydrolyse lui-même. On voit bien la nécessité de mieux connaître l'organisation aux plans physique et physicochimique de ces milieux complexes, résultante des nombreuses interactions entre les différents composants, eau, acylglycérols et autres produits. On peut en effet s'attendre à ce qu'acides gras libres et di- et monoacylglycérols dont les propriétés de surface sont bien connues, jouent un rôle majeur dans ces milieux multiphasiques ou tout au moins dispersés (micellaires). De même l'effet autocatalytique mentionné aussi dans la littérature et attribué aux acides gras libres, demande une étude approfondie pour identifier les espèces acides dissociées réellement impliquées.

Les résultats de cette thèse et ceux issus des efforts de recherche à venir permettront la mise en oeuvre effective et rentable d'une unité industrielle intégrée de bioraffinerie, jouant sur les divers avantages offerts par les fluides critiques pour (bio)raffiner la biomasse à la demande vers une gamme diversifiée de produits.

En conclusion, ce travail, nécessairement limité à un petit nombre de réactions et de composants de la biomasse, a atteint son objectif principal, en montrant la possibilité d'appliquer le concept novateur de la Bioraffinerie Intégrée en réacteur continu avec des fluides sub- ou supercritiques, contrairement à leur mise en œuvre actuelle en réacteur fermé, vers des produits commercialisables.

Dans ce domaine, étant donnée l'orientation industrielle qui a guidé le présent travail en génie des procédés, une estimation des coûts de production a été réalisée en parallèle (protocoles de calculs décrits en annexe). A titre d'exemple, les résultats montrent un coût de production des esters éthyliques de 0,45€/L (échelle de 40.000t/an). Bien que le marché actuel du biodiesel soit dominé par les esters méthyliques, les esters éthyliques obtenus au cours de ce travail constituent une alternative digne d'intérêt notamment pour la substitution du méthanol d'origine fossile par l'éthanol d'origine renouvelable. Le projet BIOscopes (Hamelinck et al., 2007), a confirmé l'intérêt de cette option en 2007 alors que le coût des esters méthyliques était estimé à 0,61€/L et celui des esters éthyliques à 0,66€/L. La possibilité qui est offerte par le procédé étudié au cours de ce travail, de remplacer les huiles raffinées ou semi-raffinées par des huiles brutes pouvant même posséder une acidité élevée, donc de coût beaucoup plus faible, est bien sûr de nature à accroître la compétitivité sur le marché des biocarburants des esters éthyliques ainsi produits, alors que les procédés actuels plus classiques, basés sur la catalyse basique ne peuvent accepter de tels substrats.

Ce travail illustre donc l'intérêt des fluides critiques non seulement au plan technique, mais aussi au plan économique, par exemple pour la production d'esters éthyliques comme biodiesel, sans oublier que ces spécialités chimiques possèdent déjà un marché en cosmétique où ils sont encore mieux valorisés.

GENERAL INTRODUCTION

This work addresses the issue of Integrated Biorefinery by developing discrete units with the ultimate objective of coupling them to enable a continuous flow configuration. Due to the complexity of biomass and the fact that biomass is most often solid there is a need for a sustainable and environmentally friendly pre-treatment process or technology that will enable any biomass or organic waste to be fully utilised while eliminating waste streams associated with the process. Sub-critical water (SCW) and supercritical CO₂ have successfully been used as a solvent for extraction of a range of compounds, in addition to hydrolysis of natural polymers and other components (regarding water) for example triacylglycerols and hemicellulose. But in most case in bibliography and at industrial level, sub- and supercritical fluids are used in batch reactors, which impede up-scaling to achieve economically viable production of commodity chemicals and even most chemicals for industrial uses. Therefore there is a need for evaluating these environmentally benign critical fluids in continuous flow reactors.

To investigate the versatility of SCW as medium to support continuous hydrolysis, sunflower oil (triacylglycerols, TAG) and rice bran (carbohydrates) were chosen as model substrates as they possess very different physio-chemically properties therefore allowing the impact of the ion product and dielectric constant of water to be evaluated. Further, the transformation of selected primary products, to add value, was also investigated. Free fatty acids (FFAs) were biocatalytically transformed to fatty acid esters using lipase within continuous flow super critical CO₂ environment. To address the complex interplay between multiple processing parameters, response surface methodologies (RSM) were used to assist with modelling the hydrolysis and esterification reactions.

The PhD report is organized in three parts. The literature review (Part A) comprises five chapters on the main fields concerning this interdisciplinary work: oils and oleochemistry (Chapter 1); natural polymers and processing lignocellulose (Chapter 2); enzymes and industrial uses (Chapter 3); critical fluids and industrial uses (Chapter 4); biorefinery (Chapter 5). Part B describes materials and methods: analysis protocols (Chapter 6), computations for kinetics, optimisation, modelling (Chapter 7), in-lab built reaction units (Chapter 8). Results are detailed in Part C. Catalyst-free hydrolysis of rice bran as model of lignocellulosic biomass, with sub-critical water (Chapter 9); catalyst-free hydrolysis of sunflower oil in sub-critical water (Chapter 10); enzymatic esterification of oleic acid with ethanol in SCCO₂ in Chapter 11, as an example of functionalisation of first reaction products into specialty chemicals. Last, Chapter 12 deals with the prediction of solubility of natural compounds in sub-critical water.

PART A: BIBLIOGRAPHY

Chapter 1

OILS & FATS

Introduction

Oils and fats are composed of triacylglycerols, (TAG), which are in turn composed of glycerol esterified with fatty acids (FAs). The carbon chain length of the FA and its degree of unsaturation determines the chemical, physical and nutritional properties of the oil or fat. Oils and FAs are used in a variety of edible and industrial applications including shortenings, margarine, emulsifiers, cosmetics, pharmaceuticals (drug carriers), soaps and detergents, paints and varnishes (drying oils) and lubricants.

1.1 Sources of oils and fats

Oils and fats are sourced from mammalian, fishes or oilseed bearing plants. Vegetable oils are sourced from predominately seeds and fruits (e.g. cotton, rapeseed, olive, coconut). Table 1.1 indicates the main oils and fats used in both edible (cooking oil, margarine) and industrial (lubricants, paints, varnishes) applications. Worldwide, palm, sunflower, soy bean and rape are the main crops grown for oil (Hammond, 1991).

Table 1.1 Edible or non-food users of oils and fats

Edible Oils		Industrial Oils
Butter	Peanut	Castor
Palm	Rapeseed	Palm
Coconut	Safflower	Lard
Corn	Sesame	Linseed
Cottonseed	Soybean	Rapeseed
Lard	Sunflower	Palm Kernel
Olive	Tallow	Tallow

(Hammond 1991)

The world's vegetable oils/animal fats industries have been grown rapidly over the last few decades. The world oil seed production is presently around 300 million tonnes/annum, providing us with around 100 million tonnes of vegetable oils such as palm, rapeseed, soybean, peanut, olive and sunflower oil (Rooney, 1997). The fats and oils production has been growing rapidly over the past few decades, far beyond the need for human nutrition. The world's production and consumption of natural oil and fats have grown from 79.2 million tonnes in 1990 to 117 million tonnes in 2001 (Gervajio, 2005). This indicates that the oils and fats industries are crucially important to the world's economy. Figure 1.1 illustrates the production of seed oil within the European Union (E.U.). Rapeseed oil is the largest oil production and has been almost stable over the period from 1998 to 2003 (United States Department of Agriculture; (USDA, 2009).

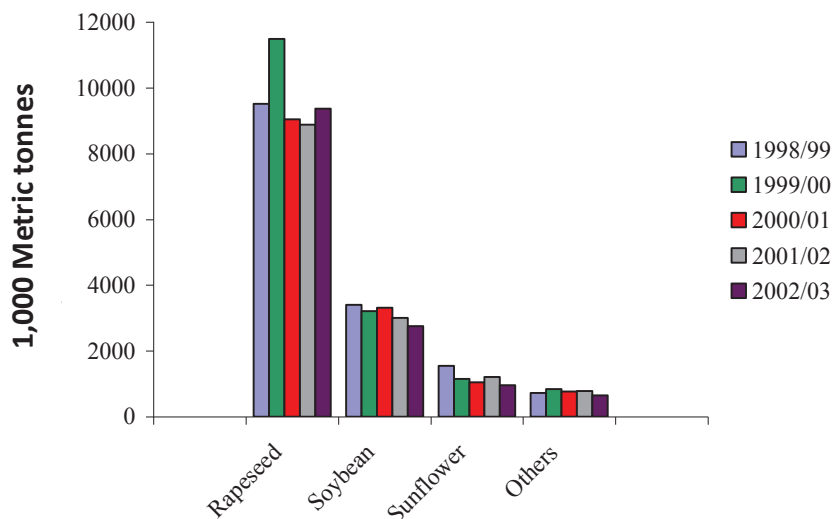


Figure 1.1 Oil seeds production within European Union

1.2 The chemistry of oils and fats

Oils and fats are chemically similar. The only difference is that oils are liquid at room temperature; fats are solid. In this section a description of their structural, physical and nutritional properties is presented.

1.2.1 Structure

Fats and oils share a common molecular structure, which is represented by the formula Figure 1.2.

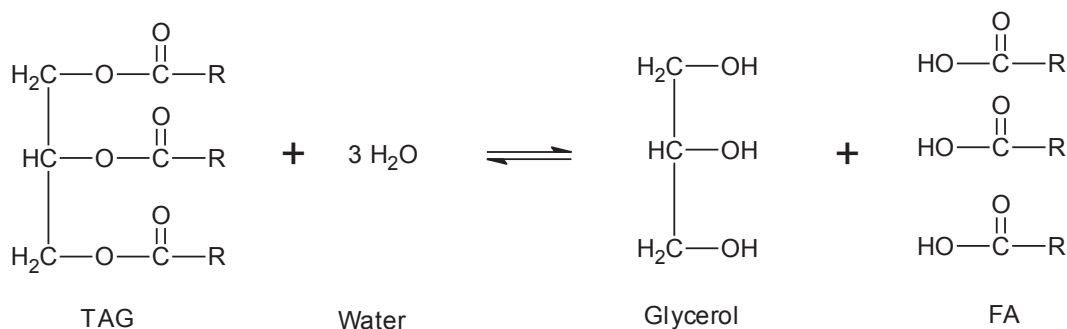


Figure 1.2 Hydrolysis of TAG (Swern, 1982)

The structural formula shown in Figure 1.2 illustrates that the oils contain three ester functional groups. They are esters of the tri-alcohol, glycerol (or glycerine), and are therefore named triacylglycerols (TAG). One of the reactions of TAG, used in industry, is the hydrolysis of the ester groups (Figure 1.2). The hydrolysis reaction produces glycerol and fatty acids, which are carboxylic acids with a normal (unbranched) hydrocarbon chain of between 5 to 35 carbon atoms. The hydrolysis reaction of TAG is catalysed by acids and bases. When a strong base such as NaOH is used, products of hydrolysis of TAG are FA salts also called soaps. Soaps are the sole

products when NaOH is used stoichiometrically e.g. sodium tallowate, a generic name for the mixture of soap obtained from tallow (animal fat), and sodium cocoate, obtained from coconut oil. TAG molecules contain mostly carbon and hydrogen atoms, with only six oxygen atoms per molecule. This means that fats and oils are highly reduced, in this way, similar to the hydrocarbons and like petroleum they are good fuels. Indeed, the main biological function of TAG is as a fuel. The normal human body stores sufficient energy in fat for several weeks of survival to deal with variations in the food supply. Plants also store energy as fats and oils. Oils are particularly common in seeds, where the stored energy helps seedlings during germination, until they can exploit solar energy through photosynthesis.

Fatty acids contain an even number of carbon atoms, from 6 to 36, bonded in an unbranched chain. Most of the bonds, if not all, between carbon atoms are single bonds. If all C-C bonds are single bonds, the fatty acid is said to be saturated. If some of the bonds between carbon atoms are double bonds, then the fatty acid is said unsaturated (Figure 1.3). When there is only one double bond, it is usually between the 9th and 10th carbon atoms in the chain, where the carbon atom attached to the oxygen atoms is counted as the first carbon atom. If there is a second double bond, it usually occurs between the 12th and 13th carbon atoms, C=C bonds being generally separated by one CH₂ (non conjugated C=C).

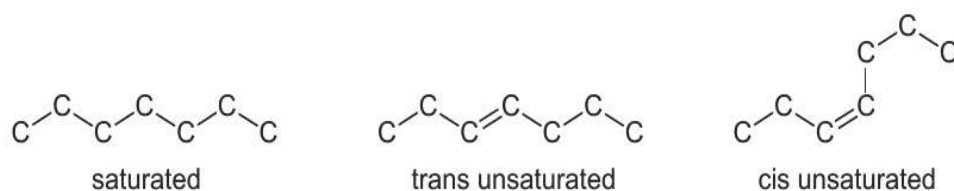


Figure 1.3 Saturated and unsaturated hydrocarbons chains of fatty acids

Double bonds between carbon atoms in FAs can cause kinks in the chains. This is particularly true for cis double bonds and these kinks can impede the molecules from stacking together well. Because they do not fit together well, unsaturated FA and TAG have lower melting points than saturated ones. Unsaturated oils undergo oxidation in the presence of oxygen and light. This multiple step reaction may cause TAG to become linked by oxygen atoms. After formation of this dimer, further linking from molecule to molecule in highly unsaturated oils causes them to solidify. Oils that undergo this process are called drying oils and are used in paints and varnishes (linseed and Tung oils).

1.2.2 Physical properties

Depending upon the fatty acid composition, fats and oils behave very differently in terms of

melting temperance and stability (Swern, 1979; Wan, 1991).

Thermal properties

The type of FAs and their distribution on the glycerol backbone determine the complex thermal behaviour and characteristics of oils. A broad measure of TAG properties regarding unsaturation is the iodine value (the weight of iodine added to C=C per 100 g of oil, whatever the FA bearing them) which goes from zero up to about 200 for the most unsaturated oils (Table 1.2).

Table 1.2 Typical fatty acid levels in olive oil and lard % weight

Fatty acid	Soybean	Maize	Palm	Olive	Lard
Palmitic acid (C16:0)	11	11	42	15	40
Oleic acid (C18:1, n-9)	23	25	38	75	40
Iodine value	120-136	115-124	44-58	80-88	60-70

(Wan, 1991)

Saturated oils have significantly higher melting temperatures. Olive oil, for example, contains on average 75% of monounsaturated (oleic) acid and only 15% of saturated FAs and is therefore liquid at room temperature (Table 1.2), whereas lard which contains about 40% of saturated FAs (palmitic, stearic), and only 40% of monounsaturated (oleic) FAs, consists of both a liquid phase and a solid phase of crystalline TAG at room temperature, the ratio of which controls the plasticity of the mixture (Swern, 1979). Furthermore, the solid phase is polymorphic, resulting in a variety of possible crystal forms of which the β' structure is considered the most desirable with respect to texture. It is the FA distribution on the glycerol backbone that determines the type of crystal structure. The crystal properties of a fat are particularly important in chocolate; the solid fat content must be of the correct crystalline structure and melt below body temperature so as to avoid a 'waxy' sensation on the palate, yet be solid enough to handle. Cocoa butter exhibits such desirable 'mouth-feel' properties and is used widely within the food industry for this reason.

Stability

Oils that have a high percentage of unsaturated FAs i.e. contain one or more double bonds, are prone to producing off-flavours and a characteristic rancid smell via either chemical, or enzymatic induced oxidation (Erasmus, 1993). The degree of unsaturation (iodine value) of the FAs present in the oil especially the content of polyunsaturated ones- determines the overall oxidative stability. However depending on the extraction and refining process naturally occurring antioxidants such as tocopherols (vitamin E) and polyphenols may improve oxidative stability.

1.3 The modification of oils and fats

Oils and fats must be extracted from mammalian, marine or vegetable source either by cooking, pressing or with a solvent (Erasmus, 1993; Swern, 1982; Wan, 1991). After processing, the oil or

fat may be refined or modified to increase its utility, in the second case *via* a range of catalytic and biocatalytic mediated reactions for example hydrogenation, hydrolysis, alcoholysis, interesterification or fractionation of the TAG. It might even be changed through genetic modification of the oil bearing fruit/seed (Erasmus, 1993).

1.3.1 Hydrogenation

Total or partial hydrogenation is used to stabilise polyunsaturated TAG and to harden oils for use as margarines and shortenings. Hydrogenation involves catalysts, such as nickel or copper, for adding hydrogen into a FA double bond (Figure 1.4). Complete hydrogenation occurs after all the C=C double bonds are converted to single bonds whereas partial hydrogenation only hydrogenates a fraction of the number of C=C. The later method allows the manufacturer to fine-tune the melting characteristics of the final product. However, during the hydrogenation process, isomerisation can occur by C=C migration to new positions on the FA carbon chain or by stereoisomeriation from the natural *cis* form to the more thermodynamically stable *trans* form. *Trans* fatty acids have been increasingly associated with a variety of negative health effects (Erasmus, 1993; Stender et al., 1995); if the oil is not completely hydrogenated *trans* isomers remain in the final product. For health reason manufacturers are considering other methods of fat hardening such as blending and interesterification.

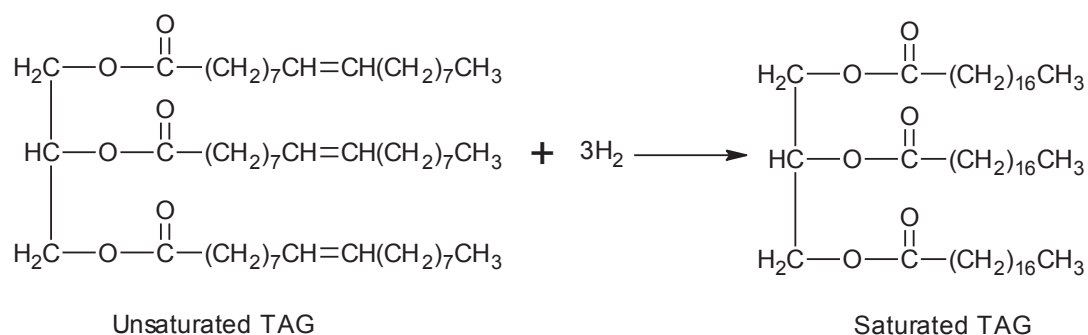


Figure 1.4 Hydrogenation of a TAG molecule

The choice of catalyst, temperature and pressure determine the extent of selectivity and isomerisation (Young et al., 1986). Indeed, such conditions allow a choice to be made as to the constituents of the final product (Hastert, 1991). Nickel deposited on a natural earth is the preferred choice at temperatures between 150 and 250°C (Swern, 1982; Young et al., 1986). The quality of the oil must also be such that minor components left in the oil after refining do not poison the catalyst (under their working conditions (Young et al., 1986) noted that 1 ppm sulphur poisons 0.004% nickel).

1.3.2 Hydrolysis (fat splitting)

Hydrolysis (Figure 1.2) is the reaction where FAs are cleaved from the glycerol backbone of the TAG. Partial hydrolysis results in the production of di- and monoacylglycerols which find application in the food processing industry as emulsifiers. Complete hydrolysis generates glycerol and FFA. FA and their derivatives are used in a wide range of products including soaps and detergents, candles, plasticisers, polymers, alkyd resins, lubricating greases, rubber tyres, cosmetics, polishes (Swern, 1982).

Hydrolysis is carried out by contacting the oil with water. The highest rates of TAG hydrolysis are obtained when the system is homogenous (Swern, 1982). Rate of the hydrolysis reaction can be enhanced by the addition of a catalyst such as mineral acids, metal oxides. The reactions are carried out either as a batch or continuous process at low (atmospheric), moderate (1 – 3.5MPa) or high pressure (5MPa) and at temperatures between 150 and 280°C (the desired quality of the product will determine the choice of process conditions). Industrial conditions are: no catalyst, 250°C and 5 MPa).

1.3.3 Esterification

Esterification is the reverse of hydrolysis. It involves a reaction in which a FA combines with an alcohol group such as methanol or glycerol (Figure 1.2). Particular esters derived from such a reaction are useful as emulsifiers, cosmetics, solvents, plasticisers, lubricants and quick drying protective coatings, and diesel fuel (Swern, 1982). The chemical reaction can occur in the absence of a catalyst at an elevated temperature (200-270°C) under reduced pressure to continuously remove water produced by the reaction and shift the equilibrium. Acid metal catalysts such as finely divided zinc and tin can be used and offer improved rates of reaction at lower operating temperatures, but uncatalysed esterification reactions are used to produce lighter coloured oils without the need to bleach. Although considerable research has been carried out on the use of enzymes (lipases) for esterification and hydrolysis they are not widely used commercially; their application lies more within the area of interesterification and transesterification for the production of structured fats and oils in the food.

1.3.4 Transesterification

In the transesterification of vegetable oils, a TAG reacts with an alcohol in the presence of a strong acid or base, producing a mixture of FA alkyl esters and glycerol (Schuchardt et al., 1995) (Figure 1.5). The overall process is a sequence of three consecutive and reversible reactions, in which di and monoacylglycerols are formed as intermediates. The stoichiometric reaction requires 1 mole of TAG and 3 moles of alcohol. However, an excess of the alcohol is used to shift the

equilibrium, increasing the yield of alkyl esters and to allow its phase separation from the glycerol formed. Several aspects, including the type of catalyst (alkaline or acid), alcohol/TAG oil molar ratio, temperature, purity of the reactants (mainly water content) and FFA content have an influence on the course of the transesterification (Schuchardt and Vargas, 1998).

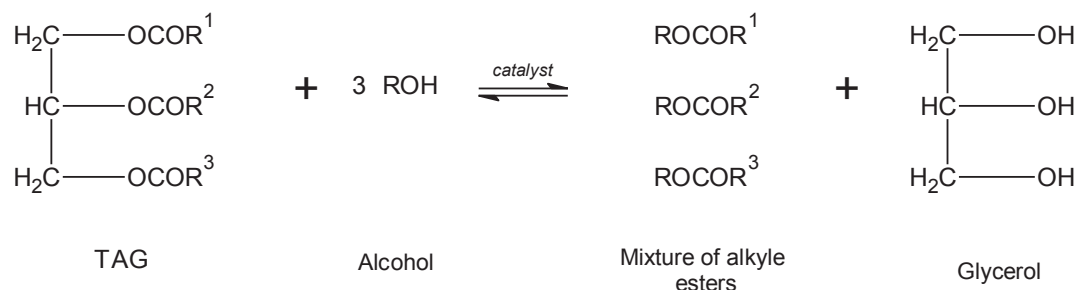


Figure 1.5 Transesterification reaction

Chemical catalysis

Alkali metal hydroxydes or alkali metal alcoholates such as sodium hydroxide or sodium methylate may be used as chemical catalysts. These reduce the operating temperature range to between 25 and 100°C and allow random interesterification (Young et al., 1986). Randomisation is useful in changing the melting characteristics of the oil, and in changing or stabilising the type of crystal formed by the TAG (Borges and Díaz, 2012; Young et al., 1986).

Enzymatic catalysis

Enzymatic catalysis reduces operating conditions usually to room temperature. They are used to provide either random or specific interesterification, depending upon the choice of lipase (Chapter 3). Sn-1, sn-3-specific and sn-2-specific enzymes (relative to the glycerol backbone) allow the specific positional replacement of FAs on the glycerol backbone. This allows the production of structured fats which have been shown to be useful in a variety of health food products. Non-specific enzymes also exist and, like chemical catalysts, allow randomisation.

In the UK, fats produced by enzymatic interesterification are currently accepted as an ingredient in chocolate and in frying fats (up to 20% total fat), provided that saturated fat intake does not increase and that the enzyme used has been cleared for use in food (Health, 1993). Structured TAG constructed through the enzymatic intraesterification of oils containing long and medium chain fatty acids have also been used to treat animals with burn injuries, critically ill patients and those suffering from Cystic Fibrosis. Such chemically modified fats have been shown to provide significant benefits over fat blends (two or more fats mixed together) made up of the same constituent oils (Bell et al., 1997b), like structured TAG in substitute infant milk formulas to mimic more closely those TAG found in human breast milk (Bell et al., 1997b).

Chapter 2

NATURAL POLYMERS

Introduction

We will consider here the natural polymers relevant to the aim of the PhD, i.e. an exploration of several options for shifting industrial chemistry from fossil source to a renewable one, therefore the following discussion is oriented towards lignocellulose a potentially valuable source of carbohydrates.

Carbohydrates consist of carbon, hydrogen and oxygen, the last two in a ~2:1 ratio, with the general formula $C_m(H_2O)_n$ and are viewed as hydrates of carbon, hence their name (Ward and Derek, 1972). As one of the major groups of naturally occurring organic compounds, carbohydrates have many important industries, including manufacture of sugar and sugar products, D-glucose and starch products, paper and wood pulp, textile fibres, plastics, foods and drinks, fermented products including beverages and ethanol for fuel, and to a less developed extent, pharmaceuticals, drugs, and specialty chemicals like surfactants (Nitschke and Costa, 2007). This chapter will review the sources of carbohydrates, their structure especially lignocellulosic materials, and will focus then on the many ways that have been proposed and used for industrial processing of lignocellulose into chemicals or fuels.

2.1 Sources of carbohydrates

There are two types of carbohydrates 1) simple carbohydrates which comprise monosaccharide (glucose, fructose, galactose) and disaccharides (sucrose, lactose, maltose) and are highly water soluble and 2) complex carbohydrates which are represented by oligomeric and polymeric forms of simple carbohydrates. e.g. hemicelluloses, cellulose and starch (Rhodes, 1995).

Hemicellulose and cellulose are naturally abundant organic material which occur within a lignocellulosic complex with lignin and serve as a structural material. Table 2.1 list the various sources of the lignocellulosic material. As an indication in Europe around 150-184 million metric tons lignocellulosic material are produced-processed annually (Macchetta, 2006).

2.2 Structure of lignocellulosic biomass

The carbohydrates comprise several homologous series characterised by a plurality of hydroxyl groups and one or more functional groups, particularly aldehyde or ketone groups, usually in the hemiacetal or acetal forms. Natural polymers of simple sugars, having acetal linkages joining the component residues, are a very important portion of the carbohydrate group, known as oligo- and polysaccharides.

Lignocellulosic biomass is the most abundant material in the world (Ghosh and Prelas, 2011). Sources range from trees to agricultural residues. Historically lignocellulosic materials were used as firewood, building materials and animal food. However, today a large industry has developed

around the utility of cellulose a major chemical constituent of lignocellulosic biomass providing paper, pulp and chemicals.

2.2.1 Main components of lignocellulose

Lignocellulose is a principal component of plant cell walls, and is composed of cellulose, hemicellulose, and lignin, along with smaller amounts of pectin, protein, extractives (soluble non-structural materials such as non-structural sugars, nitrogenous material, chlorophyll, and waxes)(Jørgensen et al., 2007). The ratio of these constituents can vary from one plant species to another. For example, hardwood has greater amounts of cellulose, whereas wheat straw and leaves have more hemicellulose (Table 2.1) (Sun and Cheng, 2002b). In addition, the ratios between various constituents within a single plant vary with age, stage of growth, and other conditions (Perez et al., 2002).

Table 2.1 Cellulose, hemicellulose and lignin content in common agricultural residues

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood Stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulps	60-70	10-20	5-10
Primary waste water solids	8-15	n.a	n.a
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal bermudagrass	25	35.7	6.4
Switch grass	45	31.4	12
Swine waste	6.0	28	n.a

(Jørgensen et al., 2007)

2.2.2 Cellulose

Cellulose is the main structural constituent in plant cell walls and is found in an organised fibrous structure, made of numerous single glucose polymer chains (Figure 2.1).

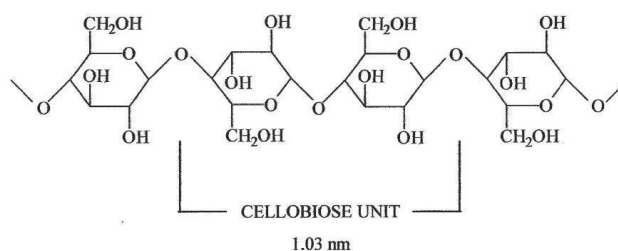


Figure 2.1 Schematic illustration of a cellulose chain

This linear polymer consists of D-glucose subunits linked to each other by β -(1,4)-glycosidic bonds. Cellobiose is the repeat unit established through this linkage, and it constitutes cellulose chains. The long-chain cellulose polymers are linked together by hydrogen and van der Waals bonds, which cause the cellulose to be packed into microfibrils. Cellulose in biomass is present in both crystalline and amorphous forms. Crystalline cellulose comprises the major proportion of cellulose, whereas a small percentage of unorganised cellulose chains form amorphous cellulose. Cellulose is more susceptible to enzymatic degradation in its amorphous form (Beguin and Aubert, 1994). Fermentable D-glucose can be produced from cellulose through the action of either acid or enzymes breaking the β -(1,4)- glycosidic linkages.

2.2.3 Hemicellulose

Hemicellulose and lignin cover the microfibrils of cellulose. The main feature that differentiates hemicellulose from cellulose is that last has branches with short lateral chains consisting of different sugars. These monosaccharides units include pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids (e.g., 4-omethylglucuronic, D-glucuronic, and D-galactouronic acids). The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by β -(1,4)-glycosidic bonds and occasionally β -(1,3)-glycosidic bonds (Kuhad et al., 1997). Also, hemicelluloses can have some degree of acetylation, for example, in heteroxylan. In contrast to cellulose, the polymers present in hemicelluloses are easily hydrolysable. These polymers do not aggregate, even when they co-crystallize with cellulose chains.

2.2.4 Lignin

Lignin is a complex, large macromolecular structure containing cross-linked polymers of phenolic monomers (Figure 2.2). It is present in the primary cell wall, imparting structural support, impermeability, and resistance against microbial attack (Perez et al., 2002). Three phenyl propionic alcohols exist as monomers of lignin: coniferyl alcohol (guaiacyl propanol), coumaryl

alcohol (p-hydroxyphenyl propanol), and sinapyl alcohol (syringyl alcohol). Alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds link these phenolic monomers together. In general, herbaceous plants such as grasses have the lowest contents of lignin, whereas softwoods have the highest lignin contents (Table 2.1).

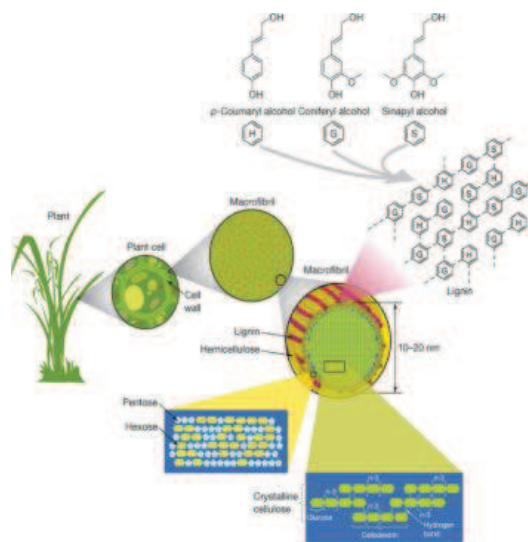


Figure 2.2 Structure of lignin (plantoils.in;2008)

2.3 Conversion of lignocellulosic biomass

Lignocellulosic feedstocks offer a significant amount of carbohydrates that have largely been untapped for conversion into value-added products via chemical and/or biological processes. The difficulty in liberating these carbohydrates from biomass materials in a cost-effective manner has been the primary hindrance to their widespread utilisation in commercial processes (Richard, 2001). In comparison, starch-based carbohydrates have been used extensively in a variety of food, chemicals, and ethanol production for energy, as efficient processes exist to liberate these carbohydrates in useable forms at high yields. With lignocellulosic feedstocks significantly less costly than starch-rich feedstocks, it is reasonable to assume that the development of efficient processes to depolymerise lignocellulosic materials could greatly facilitate the growth of a lignocellulosic-based industry for a variety of products.

Numerous methods have been investigated over the years for the total and/or partial depolymerisation of carbohydrates in lignocellulosic biomass, including a variety of thermal, chemical, and mechanical processes, and combinations thereof. Due to the wide heterogeneity of biomass materials, no one single process has been widely accepted as the optimum process. These are reviewed hereafter. We kept the name of “pre-treatment” according to the generally accepted view although we will later show a different position taken by the industrial partner of this PhD work.

2.3.1 Pre-treatment of lignocellulosic materials

Goals of pretreatment

The beneficial effects of the so called pre-treatment of lignocellulosic materials have been recognised for a long time (McMillan, 1994). The goal of a pre-treatment step is to increase the overall porosity of the lignocellulosic materials by removing lignin and hemicellulose and reduce the crystallinity of cellulose. Pre-treatment must meet the following requirements:

- (1) Improve the accessibility to sugars to support efficient downstream utilisation e.g. fermentation
- (2) Avoid degradation or loss of carbohydrate
- (3) Avoid the formation of by-products that are inhibitory to the subsequent hydrolysis and fermentation processes, and
- (4) Be cost-effective.

A general literature survey suggests that pre-treatment methods can be roughly divided into the following categories, and the most promising and cost effective for bioconversion are then discussed:

- physical (milling and grinding), physicochemical (steam pre-treatment/autohydrolysis, hydrothermolysis, including wet oxidation),
- chemical (alkali, dilute acid, oxidizing agents, and organic solvents),
- biological, electrical, or a combination of these.

2.3.2 Physical pretreatment

Mechanical comminution

Comminution of lignocellulosic materials through a combination of chipping, grinding, and/or milling can be applied to reduce cellulose crystallinity. The size of the materials is usually 10-30 mm after chipping and 0.2-2 mm after milling or grinding (Sun and Cheng, 2002b). Vibratory ball milling was found to be more effective than ordinary ball milling in reducing cellulose crystallinity of spruce and aspen chips, and in improving their digestibility (Millet et al., 1976). The final particle size desired, and the biomass characteristics determine the power requirement for mechanical comminution of agricultural materials (Cadoche and López, 1989). Physical pre-treatment is often used in combination with other treatment in order to facilitate the appropriate breakdown of biomass.

Pyrolysis

Pyrolysis has also been used for the pre-treatment of lignocellulosic materials. Cellulose rapidly decomposes to gaseous products and residual char when biomass is treated at temperatures above

300°C (Kilzer and Broido, 1965; Shafizadeh and Bradbury, 1979). At lower temperatures, the decomposition is much slower, and the products formed are less volatile (Bridgwater, 2006).

2.3.3 Physicochemical pretreatment

Steam explosion

Steam explosion is the most commonly used method for the pre-treatment (McMillan, 1994). Biomass is treated with high-pressure saturated steam, and then the pressure is suddenly reduced, which makes the materials to undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160-260°C (corresponding pressure, 0.69-4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure (Sun and Cheng, 2002b). The biomass/steam mixture is held for a period of time to promote hemicellulose hydrolysis, and the process is terminated by an explosive decompression. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the ease of cellulose hydrolysis. Hemicellulose is thought to be hydrolysed by acetic and other acids released during steam-explosion pre-treatment (Horn et al., 2011).

Ammonia fibre explosion (AFEX)

Ammonia fibre explosion is a physicochemical pre-treatment process in which lignocellulosic biomass is exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is suddenly reduced. AFEX is very similar to steam explosion. The dosage of ammonia is 1-2 kg per kg of dry biomass, the temperature is 90 °C, and the residence time is 30 min (Venkatesh Balan, 2010).

Carbon dioxide explosion

In attempts to develop improved lignocellulose pre-treatment techniques, the idea of using supercritical CO₂ explosion, which would allow a lower temperature than steam explosion and possibly a reduced expense compared to AFEX, was developed. Supercritical fluid refers to a fluid that is compressed above its critical point to a liquid like density. It was hypothesised that, CO₂ forms carbonic acid when dissolved in water, thus acting as a catalyst and increasing the hydrolysis rate. Carbon dioxide molecules are comparable in size to water and ammonia and should be able to penetrate small pores accessible to these molecules. CO₂ was suggested to be helpful in hydrolysing hemicellulose as well as cellulose. Moreover, the low temperature prevents any appreciable decomposition of monosaccharides by the acid. Upon an explosive release of the pressure, the disruption of the cellulosic structure increases the accessible surface area of the substrate to hydrolysis (Zheng et al., 1995).

2.3.4 Chemical pre-treatment

In addition to above chemical effects combined to mechanical action of fast pressure release, other chemical steps have been proposed.

Ozonolysis

Ozone treatment is one way of reducing the lignin content of lignocellulosic wastes and results in an increase of the in vitro digestibility of the treated material, and unlike other chemical treatments, it does not produce toxic residues. Ozone can be used to degrade lignin and hemicellulose in many materials such as wheat straw (Ben-Ghedalia and Miron, 1981), bagasse, green hay, peanut, pine (Neely, 1984), cotton straw (Ben-Ghedalia and Shefet, 1983), and poplar sawdust (Vidal and Molinier, 1988). The degradation is mainly limited to lignin. Hemicellulose is slightly affected, but cellulose is not.

Acid hydrolysis

Concentrated acids such as H_2SO_4 and HCl have also been used to treat lignocellulosic materials. Pre-treatment with acid hydrolysis can result in improvement of enzymatic hydrolysis of lignocellulosic biomasses to release fermentable sugars. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive, hazardous, and thus require reactors that are resistant to corrosion, which makes the pre-treatment process very expensive. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sun and Cheng, 2002b; von Sivers and Zacchi, 1995).

Alkaline hydrolysis

Some bases can be used for the pre-treatment of lignocellulosic materials, and the effect of alkaline pre-treatment depends on the lignin content (Fan et al., 2007; McMillan, 1994). Alkali pre-treatment processes utilize lower temperatures and pressures than other pretreatments (Mosier et al., 2005). Alkali pre-treatment can be carried out at ambient conditions, but pre-treatment times are on the order of hours or days rather than minutes or seconds. Compared with acid processes, alkaline processes cause less sugar degradation, and many of the caustic salts can be recovered and/or regenerated. Sodium, potassium, calcium, and ammonium hydroxides are suitable alkaline pre-treatment agents (Sun and Cheng, 2002a).

Liquid hot water pretreatments

Water pre-treatments use pressure to maintain the water in the liquid state at elevated temperatures (Bobleter, 1994; Bobleter et al., 1981; Bobleter and Concini, 1979; Bobleter et al., 1976; Hörmeyer et al., 1988; Kohlmann et al., 1996; Mok and Antal, 1992; van Walsum et al., 1996; Walch et al., 1992). Flow-through processes pass water maintained in the liquid state at elevated temperatures through cellulose. This type of pre-treatment has been termed hydrothermolysis (Bobleter et al.,

1981; Bobleter and Concin, 1979), aqueous or steam/aqueous fractionation (Bouchard et al., 1991), uncatalysed solvolysis (Mok et al., 1994; Mok and Antal, 1992) and Aquasolv (Allen et al., 1996). Solvolysis by hot compressed liquid water uses contact times up to 15 min at temperatures of 200-230°C. Between 40% and 60% of the total biomass is dissolved in the process, with 4–22% of the cellulose, 35–60% of the lignin and all hemicelluloses being removed. Over 90% of the hemicellulose is recovered as monomeric sugars when acid is used to hydrolysis the resulting liquid. The pre-treatment results were found to be virtually independent of temperature and time. Variability in results was related to the biomass type with high lignin solubilisation impeding recovery of hemicellulose sugars (Mok et al., 1994; Mok and Antal, 1992). There are three types of liquid hot water reactor configurations. Co-current, counter-current, and flow through. In co-current pretreatments, a slurry of biomass and water is heated to the desired temperature and held at the pre-treatment conditions for a controlled residence time before being cooled. Counter-current pre-treatment is designed to move water and lignocellulose in opposite directions through the pre-treatment reactor. In a flow-through reactor, hot water pass over a stationary bed of lignocellulose, hydrolyses and dissolves lignocellulose components and carries them out of the reactor.

Oxidative delignification

Lignin degradation can be catalysed by the peroxidase enzyme in the presence of H₂O₂ (Azzam, 1989). Wet oxidation combined with base addition readily oxidizes lignin from wheat straw, thus making the polysaccharides more susceptible to enzymatic hydrolysis. Furfural and hydroxymethyl furfural, known inhibitors of microbial growth when other pre-treatment systems are applied, were not observed following the wet oxidation treatment.

2.3.5 Other pre-treatment methods

Pulsed-electric-field pre-treatment

Pulsed electric field (PEF) pre-treatment involves application of a short burst of high voltage to a sample placed between two electrodes. When an electric field is generated between two parallel-plate electrodes, the field strength (E) is given by $E = V/d$, where V is the voltage and d is the distance between the two electrodes (Vorobiev and Lebovka, 2010).

PEF pre-treatment can have serious effects on the structure of plant tissues. When a high-intensity, external electric field is applied, a critical electric potential is induced across the cell membrane, which leads to rapid electrical breakdown and local structural changes of the cell membrane, the cell wall, and therefore the plant tissue. The electric field results in a dramatic increase in mass permeability and, in some cases, mechanical rupture of the plant tissue.

2.3.6 Pre-treatment synopsis

It must be emphasised that it is not always possible to transfer the results of pre-treatment from one type of material to another. Further, one technology that is efficient for a particular type of biomass material might not work for another one (Zheng et al., 2009). Various pre-treatment processes for lignocellulosic biomass, and their advantages and disadvantages, are summarised in Table 2.2. According to (Kumar et al., 2009) the choice of the pre-treatment technology used for a particular biomass depends on its composition and the targeted products. These factors significantly affect the costs associated with a pre-treatment method.

Table 2.2 Summary of various processes used for the pre-treatment of lignocellulosic biomass

Pre-treatment Process	Advantages	Limitations and Disadvantages
Mechanical comminution	Reduces cellulose crystallinity	Power consumption usually higher than inherent biomass energy
Steam Explosion	Causes hemicellulose degradation and lignin transformation; cost-effective	Destruction of a portion of xylans; incomplete disruption of the lignin-carbohydrate matrix; generation of compounds inhibitory to microorganisms
AFEX	Increases accessible surface area, removes lignin and hemicellulose to an extent; does not produce inhibitors for downstream processes	Not efficient for biomass with high lignin content
CO ₂ explosion	Increases accessible surface area; cost-effective; does not cause formation of inhibitory compounds	Does not modify lignin or hemicellulose
Ozonolysis	Reduces lignin content; does not produce toxic residues	Large amount of ozone required; expensive
Acid hydrolysis	Hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure	High cost because of corrosion; formation of toxic substances
Alkaline hydrolysis	Removes hemicellulose and lignin; increase accessible surface area	Long residence time required; irrecoverable salts formed and incorporated into biomass
Liquid hot water	Increase accessible surface area, Auto catalyst hydrolysis	High Energy Cost
Organosolv	Hydrolyzes lignin and hemicellulose	Solvents need to be drained from the reactor, evaporated, condensed and recycled; high cost
Pyrolysis	Produces gas and liquid products	High temperature; ash production
Pulsed electric field	Ambient conditions; disrupts plant cells; simple equipment	Process needs more research
Biological	Degrades lignin and hemicelluloses; low energy requirements	Rate of hydrolysis is very low

ENZYMES AS INDUSTRIAL CATALYSTS

Introduction

In terms of industrial application enzymes are increasingly being used as a result of their ability to carry out operations much more cheaply and effectively than conventional chemical methods. Furthermore they may be used in the synthesis of compounds that previously were impossible to achieve chemically. Lipases in particular have shown significant potential for the synthesis and modification of oils (Houl, 2011).

3.1 The chemistry of enzymes

3.1.1 Definition of an enzyme

Enzymes are highly specific catalysts both in terms of the substrate and of the reaction itself. Enzymes binds substrates by a mechanism referred to as the lock and key hypothesis forming a catalytic triad (or active site) (Segel, 1975b). Thus, it will become clear from the discussion below that the primary, secondary, tertiary and quaternary structure of the enzyme determines the shape of this triad and therefore its specificity.

Enzyme structure

An enzyme is a protein made up of α -amino acids (Figure 3.1a), linked together by a peptide bond (Figure 3.1b). Unless the R group is hydrogen these acids are optically active. All nineteen types of amino acid found in nature are of the left (levo- or L) form (Bailey, 1986).

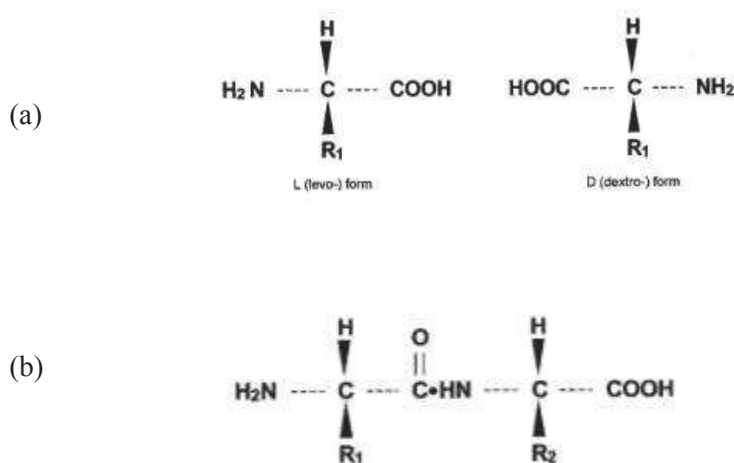


Figure 3.1 a) Alpha- amino acid stereo isomers and b) example of a peptide bond (.)

The amino acid sequence is termed the ‘primary’ structure (Price and Stevens, 1989b). The three dimensional configuration of the enzyme (determined by X-ray crystallography) consists of the secondary and tertiary structures. The former describes regular events in the sequence such as the α -helix (spiral shape, held together by hydrogen bonding) and the β -pleated sheet (flat corrugated

shape, Figure 3.2). The latter describes the overall structure. Enzymes also occasionally consist of multiple polypeptides linked together by non-covalent bonding. This is described as the 'quaternary' structure.

The active site may be determined through manipulation of the DNA sequence (the code required by the organism to build the enzyme) by deleting and inserting specific amino acids. If the deleted amino acid produces an enzyme with low activity, that acid may be assumed to be part of the catalytic triad is generally composed of serine, histidine and glutamine (Schrag et al., 1991). For the catalytic triad to be 'active', the enzyme must be in its three dimensional (folded) state. It is maintained by both “weak chemical” (hydrogen bonding, Van der Waals forces) and chemical bonding (disulphide bridges). The active structure may be lost by either physical (heat) or chemical (pH) denaturing agents. However, the primary structure appears to contain the required information to allow refolding of the enzyme (Price and Stevens, 1989b). Thus, if the primary structure is retained, once the denaturing agent is removed the enzyme will refold into its active form. The process of refolding is thought to occur through the formation of several small local structures along the polypeptide chain (like the α -helix and the β -pleated sheet), that then act as 'nucleation' centres for further refolding.

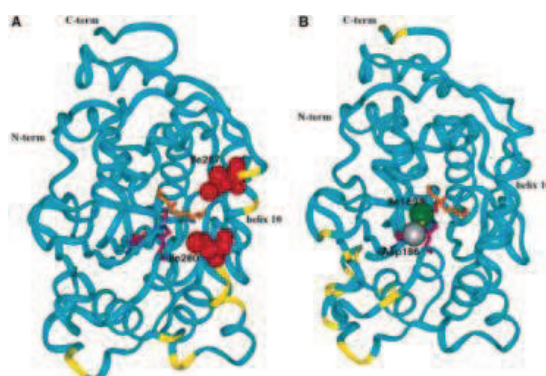


Figure 3.2 *Candida antarctica* lipase (peds.oxfordjournals.org; 2012)

3.1.2 Enzyme specificity

The size and shape of the enzyme catalytic triad together with the surrounding structure restricts the type, shape and size of molecules that can attach to the active site. Enzymes are therefore highly specific in terms of both the substrates that maybe used and the reactions that can be catalysed. For example enzymes can be stereo-specific (useful in resolving racemic mixtures) or indeed regio-specific (useful in reacting with a particular region of a substrate). Lipases in particular might be used to specifically hydrolyse the two outside fatty acids of a TAG molecule whilst retaining the fatty acid present in the middle position. Used in an industrial process such

catalytic properties offer the possibility of synthesising chemical compounds previously unobtainable by conventional chemical methods.

3.1.3 Enzyme classification

Enzymes are classified by the reaction they catalyse. In total six main groups exist (oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases). These are further divided into sub groups such as proteases and into further sub-groups that indicate the type of substrate. Using such a system allows an individual classification number to be assigned to every known enzyme.

Lipases for example are a sub-group (sub-group 1) of the hydrolase family (group 3) of enzymes that catalyse in particular the hydrolysis of lipids. They are therefore classified as E.C.3.1 enzymes.

3.1.4 Sources of enzymes

Living cells produce enzymes. They are used to perform various functions both within the cell (intracellular) and out of the cell (extracellular). Such cells may be categorised by their source: plant, animal or microbial. Microbial sources (or Protists) are single cell or unicellular organisms such as bacteria, fungi (moulds and yeasts), algae or protozoa. Plants and animals differ in that they contain many different types of cell.

In terms of industrial application, microbial sources offer the highest potential, as the methods used to produce them are easily scalable. Furthermore, as a result of their high growth rate they may easily be adapted to market demand. For these and other reasons microbial sources represent 90% of all enzymes produced industrially (Godfrey and West, 1996), and only very few species are used. In comparison plant and animal sources are restricted by government regulation within the food industry so as to minimise spread of viral diseases (Godfrey and West, 1996). However, in recent years, cloning and expression of gene coding in micro-organisms have allowed rare or valuable mammalian proteins to be produced on an industrial scale (Crabbe, 1990).

3.1.5 Enzyme extraction and purification

In the 1950s, microbial cultures were grown and the compound to be enzymatically modified was then added. Such practice implies the need to separate the product (from the fermented broth) after the reaction has taken place. Furthermore it is unlikely that the conditions during growth of the micro-organism are optimal during the biotransformation stage (Lilly, 1994). For these reasons the two stages must be separated. This requires the enzyme to be separated and purified from the fermented broth before using it for the biotransformation of the substrate.

Industrially, organisms that produce the required enzyme must be chosen in such a way that it is

easily separable. Intracellular production will require cell disruption to obtain the desired enzyme. A number of both chemical and physical techniques exist (Scawen and Melling, 1985), however, the majority may only be used to produce analytical quantities. Only recently has it been possible for this to be carried out on a large scale, usually with an homogeniser (Hethrington et al., 1971; Lilly, 1994). Indeed, (Wiseman, 1985) has produced an account of popular large scale equipment for the disruption of cells, although only if the protein is stable and re-usable does cell disruption become economically feasible. Comparatively, extracellular production of an enzyme does not require such procedures. It also offers the advantage of possible continuous production. For these and other reasons, the majority of enzymes available in bulk quantities are derived through extracellular production (Cheetham, 1985). After disruption of the cell (if required), the desired enzymes must then be separated from the broth and purified. pH, temperature and other conditions must be chosen so as to minimise loss of enzyme activity. A variety of techniques exist both for the separation of cells and cell debris (filtration, centrifugation) and purification of the extract (chromatography, ultra-filtration, precipitation) although only very few techniques are easily scalable (Price and Stevens, 1989a; Wiseman, 1985).

The final product will contain impurities such as protein and other components of the broth (the majority of enzymes are currently produced to food grade standard (Godfrey and West, 1996). The final product is usually available as a liquid with added preservatives (conforming to relevant regulations) or as a solid with bulking agents such as lactose. Good storage is essential to maintain the activity of the purified enzyme. Dry products stored in a cool environment offer the longer shelf life.

3.1.6 Inhibition and activation

Certain groups of enzymes require cofactors for activation of the catalytic triad. These are important in a biological system as they act as switches that turn the enzyme on and off. Such cofactors are usually a non-protein component such as a metal ion or an organic compound, and become an integral member in the enzyme structure (Price and Stevens, 1989a). Those cofactors that bind tightly to the protein structure are sometimes referred to as prosthetic groups. Inhibitors of enzymes can combine reversibly or irreversibly, inactivating the enzyme there are increasing number of enzymes inhibitors which incurs naturally e.g. terpenoids, stilbene, flavonoids and others containing phenolic groups (Yamada et al., 2010).

3.2 Industrial enzymes

Enzymes have been used industrially for many years in one form or another (such as carbohydrate converting enzymes required for brewing). Indeed, as far back as 800BC it is known that the

Greeks used enzymes for the production of cheese (NOVONORDISK, 1997).

Traditionally, oils and fats have been blended together to change their overall physical characteristics, however extensive research has shown that structured (modified) oils offer enhanced nutritional benefits to the patient. Whereas chemical methods provide random changes in TAG structure, lipases are much more specific (both regio- and stereo), as well as less energy intensive, operating at near ambient temperatures (such a low operating temperature improves product quality by reducing degradation). Unilever's subsidiary, Lodders CROKLAAN in the Netherlands, opened a commercial process plant in 1993 for the production of a cocoa butter equivalent from palm mid-fraction and stearic acid using an immobilised lipase, Lypozyme from Novo Nordisk A/S (Coleman and Macrae, 1980; Godfrey and West, 1996).

Within the enzymes classification given in section 3.1.5, hydrolases dominate the commercial/industrial market representing 75% of total (Godfrey and West, 1996), and these enzymes have a wide spectrum of applications e.g. leather, textile, pulp and paper, baking, detergent, starch and fructose production (Buchholz et al., 2005).

3.3 Market Information

The industrial enzyme market grows 4-5% annually and represent the total market value of about 5 billion US\$ (Laugesen, 2011). The increased demand for enzymes is attributed to their ability to carry out operations much more cheaply and effectively than conventional chemical methods e.g. the use of enzymes in the leather industry has significantly reduced effluent emissions of environmentally toxic waste (Godfrey and Reichelt, 1983).

3.4 Biocatalysis

Industrial biocatalysts offer significant environmental and economic benefits over conventional chemical methods of manufacture and processing (Bornscheuer et al., 2012). In particular their use in the synthesis and/or modification of fats and oils has allowed products previously unobtainable by conventional chemical methods to be manufactured such as cocoa butter substitute and breast milk substitute. Super-critical fluid, a green solvent, has been used for extraction for example of caffeine from coffee, perfumes and flavours from herbs and hop oils and propane deasphalting (Mukhopadhyay, 2000). Super-critical fluids may also be used both as a medium to support biocatalysis and as a means of selective purification and separation (through depressurisation) after reaction (Burguete et al., 2011). Yet, while technically possible for several years, such a process has not been carried out industrially as a result of an unclear economic advantage (due in part to the high equipment costs incurred). However, as health and environmental regulations tighten, the use of such non-toxic and non-hazardous solvents such as supercritical carbon dioxide is likely to become

much more attractive (Nalawade et al., 2006).

3.4.1 Lipase

Lipases are acyl hydrolases that exhibit somewhat different activation characteristics relative to other enzymes. Speculation suggests that a non-polar substrate interface is required to activate the lipase whereby a short, two turn amphipathic helix acts as a lid over the catalytic triad (Ser-His-Asp) and is moved away upon contact with this interface (Lawson et al., 1992; Reis et al., 2009). Immobilizing a lipase on to an inert support is therefore thought to cause activation by providing an interface between the enzyme and the substrate (Fernandez-Lafuente et al., 1998).

There are lipases which have a lid but do not exhibit interfacial activation e.g. lipase from *Pseudomonas glumae*, lipase from *Pseudomonas aeruginosa*, pancreatic lipases from coyapu and lipase from *Staphylococcus hyicus* showed interfacial activation with only some substrates and lipase from guinea-pig also do not undergo interfacial activation (Manali and Munishwar Nath, 2012).

Lipases are usually chosen depending upon their catalytic characteristics. Position specific (relative to the triglyceride backbone) lipases such as those derived from *Rhizomucor miehei*, or *Rhizopus arrhizus* are better suited towards the production of structured triacylglycerol (Brady et al., 1986; Erickson et al., 1990). Comparatively, non-specific lipases such as that from *Candida rugosa* allow randomization or complete hydrolysis (Brady et al., 1986). Lipases also exist that are specific to alcohols or fatty acids of a certain length (Langrand et al., 1990; McNeill and Sonnet, 1995) or indeed saturation (Coleman and Macrae, 1980). Lipases are also stereo-specific, allowing resolution of optically active isomers (such as ibuprofen) (Aaltonen and Rantakyla, 1991b). Specificity has also been found to vary in a near-anhydrous environment (Zaks and Klivanov, 1984) and after immobilisation (Pencreach et al., 1997). The effect of local pH upon lipase activity (referred to as the 'PH profile' of the lipase) might also be important, particularly in SCCO₂ where it is suggested that CO₂ modifies the local pH surrounding the biocatalyst, *Candida antarctica* lipase B supplied by Biocatalysts Ltd (Pontypridd, Wales, UK) is relatively insensitive to pH and was used in SCCO₂ for this reason (Steytler et al., 1991). A variety of both free and immobilized lipases have been shown to be active in SCCO₂ and other supercritical fluids (Appendix A-1). However, the majority of research has been carried out using commercially available biocatalysts as shown in Appendix A-2. Such biocatalysts have demonstrated excellent activity and stability in such a high-pressure environment.

3.5 Substrate and reaction type

The choice of substrate will determine the performance of the enzyme (Manali and Munishwar Nath, 2012; Steytler et al., 1991). Substrate and product solubility, viscosity, melting temperature,

enzyme compatibility (substrate pH, carbon length), enzyme inhibition and the desired product should therefore be considered. Indeed it is particularly important in a supercritical fluid that solubility data for both substrates and products are available over the pressure and temperature range to be investigated (Appendix A-3 - for a review of solubility data). It is essential to be aware of the respective solubility limits of all compounds present within a solvent. A variety of reactions have been conducted using SCCO₂ as the solvent as indicated in Appendix A-4. Studies that investigate the benefit, or otherwise, of a novel technology must strike a balance between simplicity and reality, interesterification for example consists of a hydrolysis step and an esterification step. It therefore seems wise to firstly consider such steps in isolation.

The project will explore the use of enzymatic catalysis in SCCO₂. In particular the use of a novel continuous process configuration is to be investigated (the use of near critical CO₂ as a diluent).

3.6 Reactor design

Reactor design involves the design of a process. It requires the identification of a reactor type (stirred tank, fluidised or fixed bed or membrane reactor), and a configuration (batch, semi-batch or continuous). Factors that must be considered when designing such a process include: reaction type, scale of production, cost of equipment, equipment life, safety and process flexibility (to allow production of other similar compounds) (Levenspiel, 1972b). Unfortunately "no neat formula can be expected to give the optimum set-up" (Levenspiel, 1972b). Presented therefore is an explanation that identifies the different types and configuration of reactors used for biocatalysis at high pressure and their effectiveness.

3.6.1 Identification of reactor type and configuration

For a reaction catalysed by an immobilised enzyme to proceed, the substrate(s) must transfer from the bulk solution to the support surface (external mass transfer) and from there diffuse into the support to the enzyme's active site (intra particle mass transfer), whereupon the substrate(s) reacts. The product(s) formed must then migrate through the support to the surface and from there to the bulk solution. The overall observed rate of reaction is therefore determined by a limiting step or a combination of all three steps. However, by choosing a suitable reactor, such limitations might be minimised.

Stirred tanks improve the diffusion properties of the medium by providing agitation, although particulates must be mechanically elastic and robust to withstand the resulting collisions. Comparatively, fixed bed reactors are smaller in volume, cheaper and simpler to fabricate but cannot provide the same degree of agitation as a stirred tank. Particulates must also be mechanically rigid and strong to withstand the pressures exerted by the fluid as it passes through the bed (the pressure

drop across the bed being a function of particle size). The majority of biocatalysis work in SCCO_2 has used batch stirred tank as a result of its 'one-pot' simplicity (useful for carrying out kinetic studies). However, fixed (packed) beds are the preferred commercial choice in terms of cost and effectiveness if external mass transfer is not limiting. Certainly a high pressure environment demands extremely high material and construction costs (fixed packed bed -glass beads) configured in a continuous mode were chosen for this project).

It is evident that in recent years the area of biocatalysis in supercritical fluids (in particular SCCO_2) has expanded considerably (Hobbs and Thomas, 2007). Although it seems questionable as to the benefits of using a supercritical fluid as a medium for biocatalysis alone, it is clear that lipases are both active and stable in such a medium. In terms of identifying a novel area of work, an investigation into new supports in SCCO_2 seems interesting. Certainly little work (Frykman et al., 1998) has been carried out using supports as polypropylene which exhibit excellent lipase activity retention in other environments. Indeed the low cost of polypropylene in particular offers significant potential in terms of industrial application (Idris and Bukhari, 2012).

SUPERCRITICAL FLUIDS

Introduction

Supercritical fluids (SCF) are substances that display liquid-like densities but gas-like transport properties, at or above their critical pressure and temperature. Furthermore such properties may be manipulated by varying the pressure and temperature of the system. This leads to the possibility of selective extraction or separation.

Super and subcritical fluids can be used as an alternative medium to organic solvents in reactions such as biomass conversion, environmental control, polymerisation and chemical synthesis. In particular their use in enzyme catalysed reactions has been investigated although at present no commercial processes are in operation (Marty and Condoret, 2001).

4.1 Gases and liquids at high pressure

Figure 4.1, is a general pressure-temperature diagram for a pure component (Smith and Ness, 1987). At the critical point **C**, the liquid and gas phases merge to form a single fluid. Such a substance is described as a super-critical fluid at any point above this critical temperature and pressure. The arbitrary term ‘near-critical’ may also be used for substances at a point near the critical region (King and Bott, 1993) within a reduced pressure and temperature range of 0.9 to 1.3).

The properties of a compound vary considerably around its critical region, as the pressure of a gas or liquid at a temperature greater than its critical value is increased from **A** (Figure 4.1) to above the critical pressure, **B**, the density increases significantly to near liquid values. As a result of these liquid-like densities, supercritical fluids offer enhanced solvation powers. As an example, Figure 4.2 illustrates the improvement with pressure in the solubility of oleic acid in carbon dioxide (Yu et al., 1992). Below the critical pressure (7.2MPa) the solubility in gaseous CO₂ is low. However, at elevated pressures the solubility limit is much improved. The effect of temperature is also significant (McHugh and Krukoni, 1986).

In addition to such liquid-like solvation powers, supercritical fluids possess gas-like transport properties, offering enhanced diffusivities and low viscosities. Typical diffusivities are around 10^{-8} m²/s (Debenedetti and Reid, 1986), whilst in liquids they are ten times lower at around 10^{-9} m²/s (Treybal, 1980).

Such low viscosities combined with the associated large densities of the fluid result in significant buoyant effects within the fluid (Debenedetti and Reid, 1986). This phenomenon allows a further improvement in the transport properties of the fluid through natural convection. Furthermore, supercritical fluids have zero surface tension, thus allowing unrestricted penetration into another phase (McHugh and Krukoni, 1986).

Such beneficial transport properties coupled with the ability to manipulate the solubility of

compounds with pressure and temperature has proved useful in recent years for a variety of industrial applications (Section 4.2). Furthermore, the ability to expel the solvent through depressurisation after processing significantly reduces the cost of solvent/product separation (compared to conventional organic solvents). This is particularly important in the food industry where stringent food standards require very low residual solvent levels (King and Bott, 1993).

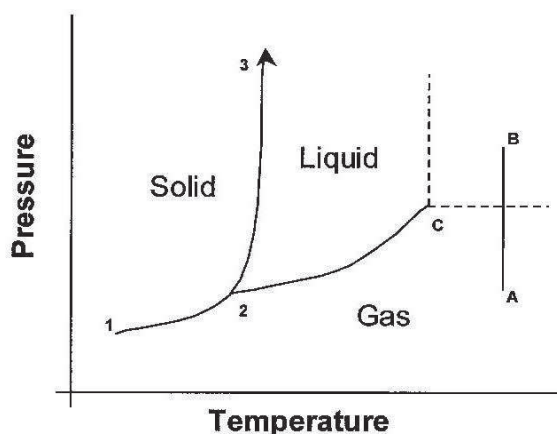


Figure 4.1 Pressure-temperature diagram for a pure compound (Smith and Ness, 1987)

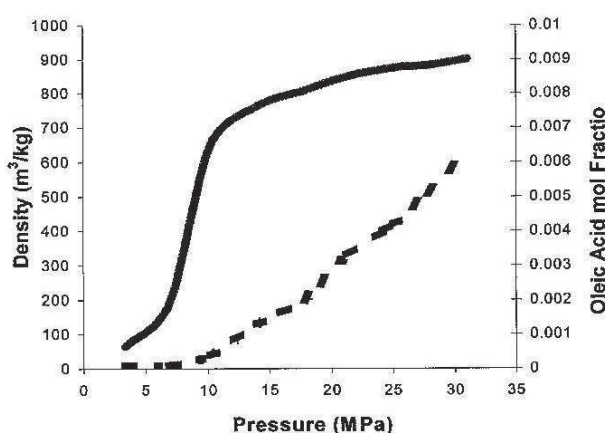


Figure 4.2 Effect of pressure on the density (----) of CO₂ and solubility (- - -) of oleic acid in CO₂ at 40°C (Yu et al., 1992)

4.1.1 Equations of state

For any process to be developed it is essential that the physical properties of the system are known or can be predicted with reasonable accuracy: an equation of state may be used to determine the physical properties of a gas/liquid (Reid et al., 1987).

The ideal gas law assumes that a gas consists of molecules that possess no finite volume and which do not interact with each other (Chang, 1981). At high temperatures or low pressures where molecules are far apart, such an assumption is valid. However, at high pressures, significant

deviations occur as molecules are brought closer together. A variety of approaches have been made to try to model such deviations. The virial equation (Equation 4-1), for example, is a series expansion and merely produces an empirical relationship from experimental data (where B, C' and D' are constants).

$$\frac{p}{RT} = 1 + \frac{B}{V} + \frac{C'}{V^2} + \frac{D'}{V^3} + \dots$$

Equation 4-1

Comparatively, cubic equations such as that first proposed by van der Waals in 1873 (Equation 4-2) try to correct the ideal gas law through a molecular interpretation (and therefore providing a physical insight into the behaviour of the gas) (Chang, 1981).

$$p = \frac{RT}{V - b} - \frac{a'}{V^2}$$

Equation 4-2

In another form it may be shown that the overall pressure of a fluid represents the sum of repulsive and attractive forces (Reid et al., 1987) The constants a' and b also have an associated physical meaning: a' is related to the magnitude of attractive forces within the gas; b is proportional to the size of the molecules within the gas (Chang, 1981). Both are related to the critical pressure, P_c and temperature T_c of the substance (Equations 4-3 and 4-4)

$$a' = \frac{27R^2T_c^2}{64P_c}$$

Equation 4-3

$$b = \frac{RT_c}{8P_c}$$

Equation 4-4

A number of other cubic equations of state have been developed in recent years. The most commonly used are the Peng-Robinson equation and the Soave-Redlich-Kwong equation (McHugh and Krukoni, 1986; Prausnitz, 1969). Such cubic equations provide an accurate representation of pressure-vapour-temperature (PVT) data over a wide pressure-temperature range and are considered more comprehensive than the Virial equation (although extrapolation should not be made outside the fitted data region, particularly at high pressures (Reid et al., 1987). Furthermore, they may also be used to predict the behaviour of both liquid and vapour phases of pure fluids (Smith and Ness, 1987). However, neither the Virial equation of state nor cubic equations of state can accurately predict fluid behaviour around the critical region. A non-

analytical component is therefore introduced that satisfies experimental data over such a region (Angus et al., 1973; Huang et al., 1985).

From Equations 4-2 to 4-4 it is evident that only the critical temperature and pressure of the gas are required to describe its PVT behaviour. This indicates that all gases behave in the same general trend (referred to as the two parameter law of corresponding states (Reid et al., 1987)). Certainly this has been found to be the case for monatomic and spherically symmetric gas molecules, however, deviations between predicted and experimental data have been observed for non-spherical and weakly polar molecules (the orientation is thus important for such molecules). A third parameter to take account of the molecular structure is therefore required (Reid et al., 1987). Different parameters have been suggested in the literature, although it is the acentric factor, ω (Equation 4-5) that is considered the most useful as it may be calculated from experimental data (Prausnitz, 1969).

$$\omega = -\log\left(\frac{P^o}{P_c}\right)_{\frac{T}{T_c}=0.7} - 1.000 \quad \text{Equation 4-5}$$

The acentric factor represents the deviation between the actual change in the reduced vapour pressure (P^o/P_c) with reduced temperature (T/T_c) and that observed for simple fluids (considered constant: 1.000 at $T/T_c = 0.7$) (Prausnitz, 1969; Smith and Ness, 1987).

In terms of accuracy, at low pressures the Virial and cubic equations of state reduce to the ideal gas law. The difference between such equations of state is therefore the complexity of the equation and the quality of the results (Reid et al., 1987) an equation of state that offers a high degree of accuracy is usually mathematically complex and requires complex physical data. A balance must therefore be made between the desire for accurate data and the complexity of the analysis.

4.1.2 Multi-component systems

Virial and cubic equations of state derived for pure fluids may be adapted to predict PVT behaviour for multi-component systems through the use of empirical mixing rules. A mixture parameter (such as the mixture's T_c or P_c) may be determined by Equation 4-6 (Reid et al., 1987).

$$E_m = \sum_k \sum_j y_k y_j E_{Kj} \quad \text{Equation 4-6}$$

The mixture parameter therefore represents the contribution of each individual component of the mixture: E_{kk} and E_{jj} represent properties of the respective pure components, where as E_{kj} is a combining term. The latter is usually calculated by taking an average of each pure component

parameter (the combining rule). A farther interaction parameter might also be introduced to allow for deviations (Reid et al., 1987). This is determined empirically and is likely to represent the differences between the two components, tending to unity for very similar components.

4.1.3 Phase equilibria

An equation of state may be used to calculate overall properties of a multi-component system such as the total pressure and total volume. However it is unable to predict partial molar quantities of the mixture (partial molar pressure, partial molar volume). Such phase equilibrium data is important for the chemical process industry for operations such as distillation, absorption and extraction (Reid et al., 1987). An accurate prediction is therefore required if data is incomplete or unavailable.

A thermodynamic analysis allows the elucidation of such parameters from PVT data (experimental or predicted from an equation of state). It is based upon the condition of thermodynamic equilibrium (Equation 4-7) between the liquid and vapour fugacities (f_k^L and f_k^V , of a component k (McHugh and Krukoni, 1986; Reid et al., 1987). This is derived through an analysis of the Gibb's free energy of a constant composition fluid at constant temperature (Smith and Ness, 1987). Put simply the fugacity of a component represents its thermodynamic partial pressure. It is analogous to the thermodynamic activity (used for liquids). Thus, it seems logical that at equilibrium the partial pressure calculated from liquid data should equal that of the partial pressure calculated from vapour data:

$$f_k^L = f_k^V \quad \text{Equation 4-7}$$

The gas and liquid fugacity, f , of a component is dependent upon the physical properties of the mixture (composition, pressure and temperature). They may be defined in a variety of forms (Reid et al., 1987), although the most straight forward technique for liquid-vapour systems at high pressure appears to be that described by (McHugh and Krukoni, 1986) (Equations 4-8 and 4-9).

$$f_k^L = X_k \phi_k^L P \quad \text{Equation 4-8}$$

$$f_k^V = y_k \phi_k^V P \quad \text{Equation 4-9}$$

In a multi-component system, the liquid and gas audacity coefficients, ϕ_k^L and ϕ_k^V , are functions of pressure, temperature and all mole fractions of the respective phase (Reid et al., 1987). They are determined using an equation of state and a departure/excess function (from the ideal state) that

allows a relationship to be made between the extensive (overall) properties of the system and the partial properties of the mixture (for an equation of state explicit in pressure, the partial molar Helmholtz free energy is applied) (Reid et al., 1987).

$$RT \ln \varphi_K = - \int_{\infty}^V \left[\left(\frac{\partial P}{\partial n_K} \right)_{T,V,n_j \neq n_K} - \frac{RT}{V} \right] dV - RT \ln Z \quad \text{Equation 4-10}$$

The fugacity coefficient for each component in the liquid and vapour phase may therefore be determined using Equation 4-10 (McHugh and Krukoni, 1986; Reid et al., 1987), with a suitable equation of state and mixing rules.

4.2 Industrial applications

4.2.1 Background

Near-critical fluids have been used in industry for a variety of extraction, separation and reaction processes over the last half century although experimentation dates back to the mid-nineteenth century (King and Bott, 1993; McHugh and Krukoni, 1986).

In the early part of the twentieth century, propane became popular as a solvent to selectively separate a lube-oil headstock into paraffin wax, asphalt, heavy ends, naphthenes, colour bodies, and the desired light oil. Known as propane deasphalting the technique used propane under conditions near its critical point to improve the separation and energy efficiency of the process (McHugh and Krukoni, 1986). Following on from the success of this technology, the development of the Solexol process in the mid-forties (concentration of polyunsaturated TAG from vegetable and fish oils) and the Residuum Oil Supercritical Extraction (ROSE) process in the early seventies then followed (McHugh and Krukoni, 1986).

It was a result of the energy crisis in the early seventies that led to a renaissance in research into the use of supercritical fluids in extraction and separation processes due to the associated energy cost savings theoretically possible (when compared with the use of conventional organic solvents). Furthermore, as a result of an increased concern for the environment in the seventies, such use of non-toxic, non-hazardous, reusable solvents like supercritical carbon dioxide (SCCO₂) found favour with the general public (McHugh and Krukoni, 1986). In particular the safety and toxicity of synthetic chemical solvents in the processing of food was questioned. This resulted in the development of several novel processes including tea and coffee decaffeinating using (SCCO₂). Such a solvent was found to exhibit excellent selectivity for caffeine, whilst allowing the essential

coffee or tea flavours to be retained. It is also physiologically harmless, cheap, easily available and non-flammable (Lack and Seidlitz, 1993). Furthermore, as legislation is introduced requiring lower and lower residual solvent tolerance levels, the use of supercritical fluids in the food industry is becoming much more economically viable. Currently SCCO₂ is used in the extraction of perfumes and flavours from herbs and hop oils; alpha acids from hops; and nicotine from tobacco (Brennecke, 1996; King et al., 2011; Robertson, 1998).

Supercritical fluids and in particular SCCO₂ are also being used for environmental applications, such as regeneration of activated carbon (McHugh and Krukonis, 1986), cleaning of precision machinery and the extraction of metals from solutions, soils and other contaminated solids (Brennecke, 1996) (of particular use in land remediation projects). Oxidation of hazardous waste using supercritical water has also been carried out on a commercial scale since 1994 (Brennecke, 1996). Super and sub-critical fluids may also be used as a replacement medium in reactions such as biomass conversion, environmental control, polymerisation and chemical synthesis (Savage et al., 1995). In particular their use in enzyme catalysed reactions has been investigated (Chapter 6), although at present no commercial processes are in operation in spite of SCCO₂ being non-toxic, non-hazardous and the ability to integrate both the reaction and separation stages of the process is of particular interest.

4.2.2 Process design

Processes using near-critical and supercritical solvents work in the pressure range of between 5 and 50 MPa, although in exceptional cases, this might be as high as 100MPa (Eggers, 1993). They are thus regarded as high-pressure processes and as a result demand high equipment costs (in terms of the capital cost of equipment, there appears to be an economy with scale, up to a limiting size (King and Bott, 1993). It is therefore important that the design a commercial supercritical fluid extraction/ separation plant is both flexible and adaptable. Indeed, the use of modular components to increase the applicability of the process seems useful (Bohm et al., 1989).

At a basic research level and indeed preparative scale, high performance liquid chromatography (HPLC) equipment e.g. high pressure tubing in a variety of diameters, fittings, high-pressure pumps and detectors (ultraviolet, infrared, refractive index) and high-pressure vessels, valves and injection mechanisms are all available off the shelf and are capable of supporting sub and super critical processes and reactions. Therefore, use of such equipment has and continues to support research in the areas of critical fluids as reflected in a wide body of literature.

The equipment required for the generation of the near- or supercritical fluid appears to be quite general. SCCO₂ for example is produced using liquid CO₂ (available in cylinders at 5MPa). It is chilled to around -10°C (to eliminate the possibility of cavitation in pump) and compressed. The

pump might be either a reciprocating or centrifugal type, the choice appearing to be dependent upon the capacity; generally for low throughputs the reciprocal pump is the preferred choice, while high throughputs demand the use of a centrifugal pump (Eggers, 1993). After the pump, the high-pressure liquid enters a further heat exchanger where upon it may be heated to above its critical temperature. The solvent is then ready for extraction/separation or reaction.

The majority of commercial extraction processes are of the batch or semi-continuous configuration (Bohm et al., 1989), whereby the supercritical fluid extracts the product as it flows through the solids bed. After separation of the product and fluid (recovery of the product is usually made through depressurisation), the solvent is recompressed, additional solvent added (to replace that lost in the separation stage) and then re-circulated through the solid bed. Such a process is repeated until the majority of product has been removed (King and Bott, 1993). The use of a liquid feed (such as palm oil) offers the potential of continuous extraction/separation (Bohm et al., 1989), although the continuous decaffeination of coffee beans using a series of 'blow cases' has also been reported (Lack and Seidlitz, 1993). The benefits of continuous processing include higher throughput and reduced labour costs. It is also likely that significantly lower quantities of supercritical fluid per unit product are required (Lack and Seidlitz, 1993).

Chapter 5

BIOMASS AND BIOREFINERY

Introduction

The transition to a more biobased production system is hampered by a variety of obstacles. Fossil raw materials are not only more economic at present, but the process technology for their conversion into organic chemicals is exceedingly well developed and basically different from that required for transforming bio-based raw materials into products with industrial application profiles.

Biomass represents an abundant carbon-neutral renewable resource for the production of bioenergy and biomaterials. Advances in genetics, biotechnology, process chemistry, and engineering are leading to a new manufacturing concept for converting renewable biomass to valuable fuels and products, generally referred to as the biorefinery. The integration of agroenergy crops and biorefinery manufacturing technologies offers the potential for the development of sustainable bioenergy and biomaterials that will lead to a new manufacturing paradigm (suschem.org, 2006).

5.1 Biomass as feed stock

Terrestrial biomass is considerably complex, constituting a multifaceted array of low and high molecular weight products: sugars, hydroxy and amino acids, lipids, and polymers such as cellulose, hemicelluloses, chitin, starch, lignin, and proteins. By far the most important class of organic compounds in terms of volume produced are carbohydrates as they represent roughly 75 % of the annually renewable biomass of about 200 billion tons. Of these, only a minor fraction (ca. 4 %) is used by man, the rest decays and recycles along natural pathways. Thus, carbohydrates, a single class of natural products - aside from their traditional uses for food, lumber, paper, and heat - are the major biofeedstocks from which to develop industrially and economically viable organic chemicals that are to replace those derived from petrochemical sources. The bulk of the annually renewable carbohydrate biomass is polysaccharides, yet their non-food utilisation is confined to textile, paper, and coating industries, either as such or in the form of simple esters and ethers. Organic commodity chemicals, however, are usually of low molecular weight, so they are more expediently obtained from low molecular weight carbohydrates than from polysaccharides. Accordingly, the constituent repeating units of these polysaccharides - glucose (cellulose, starch), fructose (inulin), xylose (xylan), or disaccharide versions thereof, most notably sucrose, are the actual carbohydrate raw materials for organic chemicals with tailor-made industrial applications: they are inexpensive, ton-scale accessible, and provide an ensuing chemistry better worked out and more variable than that of their polymers (eere.energy.gov; April 2005).

5.2 Biorefinery

Biobased economic development (bioeconomy) is nothing less than a revolution in the way society will obtain vital sources of carbon and energy, in the process dramatically reducing our dependence on petroleum/crude oil. Agriculture will make this transformation possible by providing biorenewable resources for the production of biobased products. In essence, the modern biorefinery parallels the petroleum refinery: An abundant raw material consisting primarily of renewable polysaccharides and lignin enters the biorefinery and, through an array of processes, is fractionated and converted into a mixture of products including transportation fuels, co-products, and direct energy.

The power of the biorefinery concept is supported by economies of scale and by efficient use of all incoming bioresources. A key aspect of the biorefinery concept is the imbalance between commodity chemical needs and transportation fuels. Using the petroleum industry as an illustrative example, ~5% of the total petroleum output from a conventional refinery goes to chemical products; the rest is used for transportation fuels and energy. Most visions for integrated biorefineries do not expect this ratio to change (Kamm and M.Kamm, 2004).

5.3 Biomaterials and renewable chemicals as part of biorefining concept

Biobased feed stocks are already having an impact on some practical applications, including solvents, plastics, lubricants, and fragrances. Bio-derived plastics such as polylactic acid are attracting attention, in part because of their biological compatibility and hydrolytic degradation, which enables them to successfully replace petrochemicals as well as open up new applications. Polylactic acid is currently manufactured on a thousand-ton scale in the United States and on a smaller scale in Europe and Japan (Auras et al., 2004). The production of polylactic acid is achieved by fermentation of corn dextrose to produce lactic acid that is subsequently dimerised, polymerised, and used in several applications, including food packaging and the apparel industry. The production of lactic acid by fermentation is economically competitive with its chemical synthesis from acetaldehyde and hydrogen cyanide. Further reductions in cost are expected with improvements in the fermentation process and the use of waste agricultural materials as feed stocks. Another example is the production of 1,3-propanediol by the fermentation of carbohydrates. This process is being exploited to supplement the use of petrochemically derived 1,3-propanediol to make poly(trimethylene terephthalate), a polymer fibre with properties related to Nylon and polyethylene terephthalate. The sugars in the biorefinery process can be transformed into building-block chemicals like ethanol, C3 to C6 carboxylic acids (e.g. hydroxypropanoic acid, glucaric acid), and polyols such as glycerol and sorbitol. It is noteworthy that the current cost of

many carbohydrates and their derivatives are already competitive with petrochemicals and solvents such as toluene, aniline, and acetaldehyde (Lichtenthaler, 2002).

5.4 Biofuels as part of a biorefining concept

After extracting value-added chemicals from biomass in the early stages of a biorefinery, the separations and chemical operations will need to be shifted to the production of biofuels. Today's bioethanol plant process relies largely on the fermentation of starch from corn in the United States or from sugar cane in Brazil (Lovins, 2004; Parikka, 2004). Enhancing the cost structure of bioethanol generation has moved research attention away from plant grains and more toward corn stovers, trees, and other low-cost agricultural and municipal waste materials (Shabtai et al., 2003). These biomaterials typically have higher amounts of cellulose and hemicellulose, and their efficient, cost-effective depolymerisation remains a key challenge in their use.

One important tool in reducing the cost of this depolymerisation is pre-treatment of lignocellulosics to make the biomass matrix more accessible to enzymes. The tailoring of chemical and physical pretreatments for specific biomass resources is a field of growing interest and practicality (Mosier et al., 2005). The benefits of pre-treatment are leveraged with recent research efforts that have reduced the cost of cellulase by a factor of 5 to 10.

Future cost reductions in bioprocessing will be accomplished by combining cellulase/hemicellulase treatments with other process steps. For example, researchers have proposed combining enzyme production with fermentation via modified microorganisms capable of both cellulase and ethanol production, which could provide just-in-time delivery of the optimal mixture of the hydrolytic enzymes (Zhang and Lynd, 2005).

The endogenous production of such polysaccharide hydrolyase enzymes could also be coupled with enhanced plant biomass production made possible by recent advances in molecular farming (Rishi et al., 2001). Exogenous depolymerisation enzymes used in the bioethanol process could be replaced with plants that are capable of synthesizing these enzymes in-situ. Carbohydrate depolymerase enzymes, such as cellulase, could be triggered for plant biosynthesis when an inducer is applied to the plant. A signal sequence from a cell wall protein could be spliced onto the enzyme gene to ensure that the synthesized cellulase is localized to the plant cell wall. The cellulase signal sequence-coding region would be attached to a chemically induced promoter that would switch on the enzyme gene. Once the modified transgene is introduced into a host plant, seeds could be produced, planted, and cultivated normally. Just before harvest, the crop would be sprayed with the chemical inducer. The cellulase would then be produced and transported to the cell wall, where it would start to break down the cellulose. After harvesting, the residual plant

material would be collected and transported to a biorefinery, during which the in situ-generated cellulase would continue to depolymerize cellulose to glucose. An added feature of this approach is that additional depolymerization enzymes could be brought to bear for further, no-cost conversion of plant polysaccharides to mono- or oligosaccharides, facilitating subsequent separation or fermentation operations.

Currently, the fermentation of a mixture of hexoses and pentoses is inefficient because no wild organisms have been found that can convert all sugars at high yield into ethanol. Recently, several groups have made great advances in this field by genetically modifying microorganisms (Helle et al., 2004; Lawford and Rousseau, 2002). One promising strategy has been to take a natural hexose ethanologen and add the pathways to convert other sugars. This strategy has been effective in adding pentose conversion to *Saccharomyces cerevisiae* and to *Zymomonas mobilis*. The other primary strategy has been to modify a host capable of converting multiple sugars to produce only ethanol from glycolysis. Other remaining microbiological challenges include the need to understand and manipulate ethanol and sugar tolerance, and resistance to potential inhibitors generated in pre-saccharification treatments. Solutions to these issues also will need to accommodate the variability in biomass resources.

Biological processing is not the only refining approach, however. Although biological protocols of converting polysaccharides to bioethanol are among the most developed process technologies available for biofuels, other chemical technologies are being pursued and present promising alternatives. These biofuels technologies are centred on the removal of oxygen from carbohydrates to obtain hydrocarbons.

For the biorefinery approach to be widely applicable, the lignin component of lignocellulosics must also be addressed (Chakar and Ragauskas, 2004). Residual lignin from paper pulping is now burned for heat and power, but lignin thermal-cracking studies using temperatures of $\sim 250^{\circ}$ to 600°C have demonstrated the potential of generating low molecular weight feedstocks for further processing. These high temperatures suggest that the use of cracking catalysts could lower conversion temperatures and provide improved control over product distribution. (Shabtai et al., 2003) have highlighted this potential in a process whereby a two-stage catalytic reaction with lignin produces a reformulated, partially oxygenated gasoline-like product. Lignin is first depolymerised by a base-catalyzed treatment into a series of low molecular weight phenolic compounds. This mixture is then subjected to hydroprocessing, which primarily yields a mixture of alkylbenzenes useful as a potential liquid biofuel. This pyrolysis approach to biofuels from lignin is also being pursued with biomass in general, with and without a catalyst; it provides about 58 to 77% conversion of biomass to a condensable gas, 13 to 28% non condensable gases, and 6 to

13% char formation. The condensable gases can be refined to fuels and chemicals, and the non condensables can be steam-reformed to synthesis gas (syngas), a mixture of CO and H₂, which can also be used to produce fuels and chemicals (Boateng et al., in press).

Regardless of which process technologies are incorporated into a biorefinery, almost all will generate some waste products that will be intractable and difficult to convert to value-added biomaterials or biofuels. These spent-biomass residues will contain fragments from lignin, residual carbohydrates, and other organic matter. This residue will need to be treated in an environmentally compatible manner, with the smallest ecological footprint. Such wastes and residues offer important energy sources within the biorefinery, given their chemical energy content, and are an ideal candidate for thermochemical conversion to syngas (Sricharoenchaikul et al., 2002). Syngas is an intermediate in the production of ammonia, methanol, and Fischer-Tropsch hydrocarbons. Production of syngas from coal, natural gas, and other carbonaceous sources is well established. Coal is normally gasified in entrained-flow reactors at temperatures exceeding 1,400°C at 2 to 7 MPa. Biomass is more reactive than coal and is usually gasified at temperatures between 800° and 1,000°C at 2 to 3 MPa (Baratieri et al., 2008).

The greatest challenge in producing syngas from biomass is the need to avoid poisoning the noble metal catalysts used in the subsequent downstream conversion to fuels and chemicals. Potential problem products are the alkali metals, halides, sulfur gases, and especially the tars. A high quantity of tar is produced as the organic components of biomass decompose. Evolution of tar from primary to tertiary species is rapid, but tertiary tar species are degraded slowly to CO and H₂ by water vapor or CO₂ at temperatures below 1,100°C. Catalytic conversion of tar in raw syngas to CO and H₂ is practiced, but the quantities of tar that must be converted are large, and robust catalysts that are insensitive to alkali metals, halides, sulphur, and nitrogen need to be developed. Chloride, the predominant halide in biomass, is converted to HCl or submicrometer aerosols of potassium and sodium during gasification, which poses a corrosion issue. Most of the alkali metal chlorides are removed by filtering the cooled syngas. Sulphur gases can be removed by absorption. Remaining alkali metal chlorides and sulphur gases are removed by reaction with ZnO in a packed-bed filter. Although these advances in syngas purification technologies are necessary for the catalytic conversion of syngas to other fuels or chemicals, they add further complications and increase the overall cost (Mota et al., 2011).

5.5 Sustainable processing technology for biorefinery

These commercially viable processes do, however, require purified feedstocks. The major impediment to biomass use is the development of methods to separate, refine, and transform it into chemicals and fuels.

One of these steps, separation, currently accounts for 60 to 80% of the process cost of most mature chemical processes (Laugesen, 2011). As we progress from the oil refinery to the biorefinery, the challenges associated with separation will change, but not diminish, in importance. In the petroleum industry, distillation is the unit operation that dominates the refinery separation scheme. For chemicals derived from biomass, this dominance will be transferred to solvent-based extraction. This is a result of the nonvolatile nature of most biomass components and the fact that other separation techniques, such as chromatography or membranes, do not yet have the same economies of scale.

Future biorefinery operations will first extract high-value chemicals already present in the biomass, such as fragrances, flavouring agents, food-related products, and high-value nutraceuticals that provide health and medical benefits (Morandini et al., 2005). Once these relatively high value chemicals are extracted, the biorefinery focus on processing polysaccharides and lignin into feedstocks for bio-derived materials and fuels. This requires the development of innovative separation and depolymerisation chemistries. SCCO₂, near-critical water, and gas-expanded liquids are well suited to these challenges (Eckert et al., 2004; Nolen et al., 2003). These tuneable solvents offer distinct green chemistry advantages that could be exploited in the processing of renewable bioresources (Ritter, 2004). As developed in the previous chapter, supercritical fluids exhibit outstanding transport properties coupled with highly tuneable solvent properties (such as solvent power and polarity) and ease of solvent removal. Near-supercritical fluids (or sub-critical) fluids are also highly tuneable and generally offer better transport than liquids and better solvent power than supercritical fluids.

Gas expanded liquids are mixtures of a gas with an organic liquid such as methanol or acetone; in our context, CO₂ is completely miscible with most organics. These solvents exhibit highly tuneable solvent power, as small pressure changes yield large changes in composition, and they give much greater solubility's and operate at much lower pressures than supercritical fluids. All of these solvents result in advantages for downstream processing in terms of product purification and/or catalyst recycling. Water is arguably the most environmentally benign and food-safe solvent that can be used in chemical synthesis. However, the range of water-soluble substrates is quite limited, making ambient water an unsuitable medium for many chemical syntheses. Near-critical water (200° to 300°C) exhibits a reduction in dielectric constant (20 to 30 instead of 80 under ambient conditions) and density (0.7 to 0.8 g/cm³); its ability to dissolve both non-polar organic molecules and inorganic salts is comparable to that of acetone. In addition, under these conditions, the dissociation constant of water into hydroxide and hydrogen ions rises by more than three orders of magnitude, so that near-critical water also acts as a self-neutralizing acid or base

catalyst, eliminating salt waste generation (Chamblee et al., 2004). Further, the use of near-critical water in place of organic solvents greatly simplifies product isolation, as non-polar products are insoluble after cooling. The utility of this medium has been demonstrated for a diverse group of organic syntheses (Akiya and Savage, 2002). High-temperature water has already been proposed for the depolymerisation of cellulosic waste materials in the biometrics process for producing levulinic acid (Fitzpatrick, 1996). It is noteworthy that the current cost of many carbohydrates and their derivatives is already competitive with petrochemicals and solvents such as toluene, aniline, and acetaldehyde (Lichtenthaler, 2002).

CONCLUSION OF PART A

The paradigm shift from petroleum hydrocarbons to highly oxygen-functionalized, bio-based feedstocks will create remarkable opportunities for the chemical processing industry. For example, the use of carbohydrates as chemical raw materials will eliminate the need for several capital-intensive, oxidative processes used in the petroleum industry. Biomass carbohydrates can provide a viable route to products such as alcohols, carboxylic acids, and esters, while keeping the suitable stereochemical structure, thereby reducing dependence on expensive chiral catalysts and complex syntheses that are currently required to selectively install chemical functionality in petrochemicals. The sugars in the biorefinery process can be transformed into building-block chemicals by fermentation and by enzymatic and chemical transformations. The key building block chemicals include ethanol, C3 to C6 carboxylic acids (e.g., hydroxypropanoic acid, glucaric acid), glycerol and sorbitol. It is noteworthy that the current cost of many carbohydrates and their derivatives is already competitive with petrochemicals and solvents such as toluene, aniline, and acetaldehyde. The effective production and use of these chemicals rely on the development of innovative enzymatic, catalytic green chemistries that will yield a viable range of new bio-derived products. Among these techniques, the above literature review has shown that sub and supercritical fluids, even more gas expanded liquid solvents, offer very versatile opportunities for processing biomass, either as extracting media or as reaction media. Among the key steps, so-called pre-treatment should be viewed at core of biorefinery, for loosening the fibrous macromolecular structure and facilitating extraction of small soluble –often high value- molecules and allowing deconstructing the lignocellulosic components into valuable shorter chain oligomolecules, monomers and derived compounds. In addition to more classical chemical options, enzymes offer a range of advantages, suitable for performing biomass deconstruction and functionalisation). These conclusions makes the basis of this PhD work, with the aim of exploring some of above seen opportunities for helping the merging of the biorefinery concept into a real agro- industrial production sector.

PART B: MATERIAL AND METHODS

Chapter 6

ANALYTICAL TECHNIQUES

Introduction

We used common analytical techniques e.g. titration, gas chromatography (GC) & high pressure liquid chromatography (HPLC). We have also used very interesting fast and accurate analytical technique called MicroChem II analyzer for FFA analysis. To determine the properties of hydrolysed oil we also conducted viscosity, density and pH measurements.

6.1 MicroChem II Analyzer

The MicroChem II analyser is a simple to use photometric analyser. The discrete wavelength, bichromatic photometer measures the differential absorbance of the wide variety of solutions, and converts those readings into concentration values. Bichromatic filter compartment allow maximum flexibility in wavelength selection. Wavelength are selected and changed by inserting the appropriate filter block into filter block compartment of the instrument. Samples are read directly in standard disposable tubes. The fatty acid and glycerol content of oils and samples after hydrolysis were determined using the FAsafe method.

6.1.1 Principle of analyzer

For free fatty acid

Free fatty acid (FFA) and Acid Value (AV) are determined spectrophotometrically with a chromogen reagent interacting with the FFA and changing its visible spectrum. The colour change is measured and the FFA or AV determined. This is a primary method calibrated with known amounts of FFA. The limit of detection (LOD) is 0.02%.

For total and free glycerol

Total glycerol is determined using an enzymatic reagent which digests residual glycerol and then quantifies the break down product using a chromogenic reaction which is read spectrophotometrically. Free glycerol was analysed by taking two measurements of total glycerol - one before washing the sample with distilled water and one after washing the sample. The LOD is 0.02%.

6.2 Measuring FFA concentration via titration

By way of comparison and validation of the Microanalyser, results were compared to the official titration method approved by the American Oil Chemist Society (Ca 5a-40). The protocol was used to determine the percentage of FFA in the samples collected prior to and post hydrolysis (AOCS, 1998). A sample of between 1-2 g was accurately weighed and placed in a 200 ml conical flask. 100 ml of ethanol (99.9%) was added to the sample and manually shaken until the oil had dissolved completely. In addition 2.0 ml of phenolphthalein indicator solution was added. The oil mixture was then titrated against a 0.25 N sodium hydroxide solution until a pink colour persisted

for at least 30 s. The sodium hydroxide solution was prepared by dissolving 5.0 g of sodium hydroxide pellets in 500 ml of distilled water and loaded into a 25 ml burette. The weight percentage of FFA was calculated on an oleic acid basis using equation 6-1. All samples were titrated in triplicate with a variance of < 0.5%.

$$\text{Free fatty acids as oleic, \%} = \frac{\text{ml of alkali} \times N(\text{conc. of NaOH}) \times 28.2}{\text{wt. of sample}} \quad \text{Equation 6-1}$$

where;

ml of alkali = volume of sodium hydroxide solution required during titration, ml

N(conc. Of NaOH) = concentration of sodium hydroxide, g/L

wt. of sample = weight of the sample taken, g

In order to calculate the percentage of the FFA, the units should be weight of the FFA divided by the weight of the sample times 100. This can be done by converting the 0.25 N of the NaOH from g/L to g/ml by dividing by a 1000. In order to consider the final results as a percentage, the equation 6.1 should multiply by 100.

The value of 28.2 arises from the molecular weight of oleic acid in the oil (used as reference for “C18 oils”) divided by 10. This equation was considered sufficiently accurate to determine the degree of hydrolysis.

6.3 Viscosity measurement

The rheological analysis of the FFA and sunflower oil was carried out using the AR-1000 Advanced Rheometer with controlled stress/controlled shear rate from TA Instruments (Crawley, West Sussex, UK). A sample (around 2.0 ml) of the oil/FFA was placed on the temperature controlled sample platform and analysed at 25 °C with an acrylic cone of 2° inclination (63 µm truncation) and 60 mm diameter. A solvent trap was also used. A steady state flow-curve was gathered in a linear fashion, with a shear rate ramp from 1 to 100 s⁻¹ (and 20 sample points). The viscosity was calculated as the slope of the line through data points of sheer stress plotted against the sheer rate.

6.4 Density measurement

The density measurements were conducted by a Paar DMA 35 N portable inline digital reading densitometer (Atone Paar, Eastern Petroleum Supplies LTD). It uses a mechanical oscillator technique and a thermometer to measure the temperature and density of the liquid contained

within the cell. A sample of approximately 2.5 ml was injected into the apparatus. Care was taken to prevent the introduction of small air bubbles into the cell.

6.5 pH Measurement

The pH of FFA was measured by using an inline digital reading pH meter (Seven Multi Modular Meter, Mettler Toledo, USA). The pH meter was calibrated with buffer solutions of pH 4, 7 and 10. The acid content of the samples were measured by simply dipping the indicator and the reading were noted accordingly.

6.6 HPLC based determination of carbohydrate

The analysis of carbohydrates e.g. glucose, xylose, and furfuraldehydes, HMF was conducted using high pressure liquid chromatography (HPLC). The equipment comprised: pump L-7100 (Merck-Hitachi), Rheodyne 7125i injection valve, column oven (Techlab), RI-detector (Agilent 1100), Datasystem Kroma (Bio-Tek). The column used was a Nucleogel Sugar Na, 300*7.8 mm., Eluent was deionised water, further conditioned by ion-exchange, charcoal absorbance and micro filtration to ensure removal of all bacteria. The sample volume was 20 µl, flow rate of 0.5 ml/min, column temperature 70 °C. Analytes were detected using a refractive index (RI) and calibration was done according to external standard.

6.7 Determination of total organic carbon (TOC)

To determine the total carbon content of solid samples, a Leco-2000-CNS- Analyser (Leco, USA) was used. After combustion of the sample material at 1100°C the resulting carbon dioxide was determined by means of an infrared detector. Liquid samples were analysed with the "Elementar-high TOC-Analyser". Inorganic carbon was removed by acidification prior to combustion. Detection was multi-channel-IR, and the samples were analysed in triplicate.

6.8 Free fatty acid analysis using gas chromatography

The FFA content in the hydrolysate was also determined using a gas chromatograph (HP 6890A, Agilent Technologies, USA) equipped with a DB-5 capillary column (length 30 m x diameter 0.25 mm x film thickness 0.1µm; J&W Scientific, USA) and a flame ionisation detector (FID) (340 °C). Helium was used as the carrier gas at a constant flow (2 ml/min) with a split injector (340 °C, 1:50). The oven temperature programme was 100 °C for 3 min, 10 °C/min to 150 °C, 5 °C/min to 250 °C, 10 °C/min to 350 °C, with a 15 minute hold. The samples were derivatised, to increase the volatility of the FFA and other OH containing components, by adding 1 ml chloroform, as a solvent, 10-30 mg of heptadecanoic acid, as the internal standard (a C17:0 carbon chain FFA which does not naturally occur) and 1 ml hexamethyldisilazane to ~100 mg of the sample. The

weights of the sample and internal standard were individually recorded using a four decimal place balance (Adventure –Ohaus AR-2140). The mixture was then held at 70 °C in a heating block for 50 min. The reaction mixture was cooled to room temperature before injecting (1µl) of the silylated samples into the GC column. The peak areas of all FFA components were recorded. A FFA component was identified from reference standards and its mass was calculated from a predetermined peak area response factor of the (heptadecanoic acid) internal standard. The total yield of FFA was determined by the addition of all FFA peaks. The calculated weight of FFA was then divided by the actual sample weight, to provide percentage FFA content for the samples (equation 6.2).

$$\text{FFA \%} = \frac{\text{Area of FFA peaks}}{\text{Area of internal standard peak}} \times \frac{\text{Weight of internal standard}}{\text{Weight of sample}} \times 100 \quad \text{Equation 6-2}$$

In this study, the product yield of FFA is defined as the weight percent of FFA in the final product after removal of the glycerol layer in the product mixture (equation 6.3).

$$\text{Yield of FFA\%} = \frac{\text{Weight of FFA}}{\text{Weight of product}} \times 100 \quad \text{Equation 6-3}$$

KINETIC AND STATISTICAL MODELLING

Introduction

The concept of detailed chemical kinetic modelling assists the description of complex processes with a relatively high number of intermediates, which may or may not participate in reactions. Hydrolysis reactions are important in the processing of oil and fats by the chemical industry. These reactions can be conducted thermally as a liquid-liquid reaction or gas-liquid reaction using superheated steam. They can also be conducted at ambient conditions by employing biolytic enzymes (Patil et al., 1988). The process for hydrolysing oil using water can be defined as a mass transfer controlled chemical reaction whereby water reacts with oil (triacylglycerol) to form free fatty acids (FFA) and glycerol (Lascaray, 1949). (Patil et al., 1988) have presented a three step reaction model which describes the thermal hydrolysis of different vegetable oils (peanut, tallow and coconut). Quantitative information on the reaction kinetics of non-catalytic vegetable oil hydrolysis is limited (Fujii et al., 2006; Minami and Saka, 2006); moreover, the kinetic data reported are not fully established as only one rate constant was considered for all the forward reactions.

7.1 Response surface methodology (RSM)

Process optimisation requires a multi-factorial data and data analysis. Response surface methodology (RSM) provides the tools to support process optimisation and consists of three parts

- Experimental design
- Statistical data analysis
- Modeling

The experimental designs are used to obtain a maximum of statistically significant information with the smallest number of experiments necessary when studying multiple variables. After the experimental data has been collected, it is statistically analysed using the Analysis of Variance (ANOVA). Based on the statistically significant parameters identified through ANOVA, an empirical model is fitted to the data that allows for an optimisation of the response. In this work, the software Design Expert 7.0.0 (Stat-Ease, Inc.) was used for all three steps.

7.2 Experimental Design

In an experimental design, the empirical experimental runs are chosen in a way to obtain an optimum model for navigating the design space with the least amount of experiments. The most common designs in science are full factorial designs where every possible combination of factors is run in an experiment. However, these set-ups require a large number of experiments which are often prohibitive in terms of time and cost and become impractical when more than three variables are to be studied. RSM designs reduce the number of empirical experiments required to obtain a valid data set. Another advantage of the RSM designs is the fact that the error is distributed

equally in each direction of the design space. It increases with distance from the centre of the design space, but is independent of the direction taken. One drawback with using RSM design, however, is that the results are not easily represented in two dimensional graphs as possible when a full factorial design is used. Instead, three dimensional graphs or contour plots have to be used. One of the most important experimental RSM designs is the Central Composite Design (CCD) which was used in this work. The design is shown in Figure 7.1 for the case of three variables (also called factors) studied. It consists of a cuboid with design points on each corner and one so called centre-point in the centre. The centre-point is replicated (in this case six times) in order to get information about the variation due to random error.

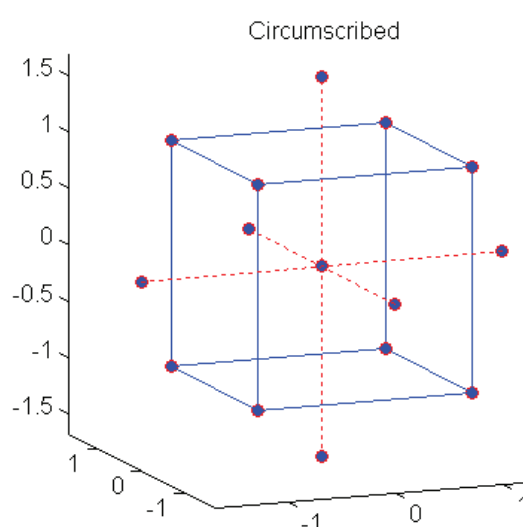


Figure 7.1 Central composite design (CCD)

Furthermore, there are six so called star points, which are located in the centre of each plane of the cuboid but reach out further. This is done in order to get information about the curvature at the border of the design space. The design space, that is the limits of the variables for which the model is produced, is only inside the cuboid and the model cannot be extrapolated.

7.3 Statistical data analysis

The ANOVA is divided into a so called 1-way ANOVA, 2-way ANOVA and N-way ANOVA, depending on the amounts of factors studied (1, 2 or >2).

The basic principle in the ANOVA is to split the data into a variation caused by the investigated level of a factor (e.g. temperature) and the variation due to random error. A value can thus be described as the sum of the mean value of the whole dataset (m), the variation of the mean due to the effect of the factor (a) and random error (e) (Geoffrey, 1991):

$$y_{ij} = m + a_i + e_{ij}$$

i	level i of investigated factor, $i=1,\dots,I$
j	data point j at level i , $j=1,\dots,J$
m	overall mean
a	level effect
e	residual

The sum of squares (SS) due to the factor levels and the residuals are calculated and the mean square (MS) is obtained by dividing with the degree of freedom, see Table 7.1

Table 7.1 Representation of ANOVA results

Source	Sum of squares	Degrees of freedom	Mean square
Factor Levels	$J \cdot \sum a_i^2$	$I - 1$	$J \cdot \sum a_i^2 / (I - 1)$
Residuals	$\sum \sum e_{i,j}^2$	$I \cdot (J - 1)$	$\sum \sum e_{i,j}^2 / (I \cdot (J - 1))$

The residual MS represents the deviation of the values at the different factor levels from the mean at that factor level whereas the factor level MS represents the deviation of the level mean from the overall mean. If there is no effect of the factor, then these two values should be similar. Dividing the level MS by the residual MS, a statistic called the F-statistic is obtained which should follow a so-called F-distribution with the degrees of freedom stated in the ANOVA table in the case of no level effect. If the F-statistic is larger than the cut-off value in the F-distribution at a given confidence level, then the factor level had an effect on the studied response. The F-test generates a p-value which states the probability that the null-hypothesis is rejected although it is in fact true (null-hypothesis: there is no difference between the two variances). Thus, low p-values are desired for significant factors. A desired significance level $1 - \alpha$ can be chosen and the factor is then considered statistically significant if $p < \alpha$.

ANOVA also provides an estimate of the magnitude of error. For example, when replicating the experiment, the results can be considered in different blocks and ANOVA will establish the variation in the results.

7.4 Modelling

By using regression analysis, the coefficients for the model terms that were found significant can be calculated. Basically, RSM uses multiple regression analysis to fit a simple polynomial to the data. Most of the time, quadratic polynomials are sufficient to describe the response and require less parameters and thus less experiments.

Finding an industrial process that requires a third-order model is highly unusual (Whitcomb, 2005). The terms used for the different types of models are shown below for the factors A, B and C. RSM models should be hierarchical, that means that if quadratic terms are included, the linear terms should be included in the model as well.

$$R = \beta_0 + \beta_1.A + \beta_2.B + \beta_3.C + \beta_4.AB + \beta_5.AC + \beta_6.BC + \beta_7.A^2 + \beta_8.B^2 + \beta_9.C^2$$

β_i	regression coefficients
R	response value
A,B,C	factors; linear terms
AB,AC,BC	interaction terms
A^2, B^2, C^2	quadratic terms

In this work, a backward regression procedure was used. The regression starts with an ANOVA of the full model specified (e.g. quadratic). Then, model terms with the highest partial p-value are removed and the ANOVA. The procedure is finished when the highest p-value of all model terms satisfied the α -out criterion (e.g. $p < 0.05$). Then, regression is used to determine the coefficients β_i of the remaining model terms. The statistics used to evaluate the goodness of the model are R^2 , R^2_{adj} and R^2_{pred} . R^2 is calculated as follows:

$$R^2 = 1 - \frac{SSE}{SST}$$

SSE	Error Sum of Squares	
y_i	Measured data value	$(\sum (y_i - \hat{y}_i)^2)$
\hat{y}	Predicted value	$(\sum (y_i - \bar{y})^2)$
SST	Total Sum of Squares	
\bar{y}	Overall mean	

$$R^2_{adj} = 1 - \frac{SSE \cdot (n-1)}{SST \cdot (v-1)}$$

n	number of response values
v	$n - m$; m = number of fitted coefficients

R^2_{adj} is R^2 corrected for the number of terms in the model, as with every model term added the R^2 will get closer to 1 without a real improvement in the model (a model with n parameters will always fit n points accurately with $R^2 = 1$). Therefore, R^2_{adj} should be maximized when choosing the model instead of R^2 . R^2_{pred} is a measure of the predictive capacities of the model, obtained by

leaving out one data point at a time during regression. As a rule of thumb, these two statistics should be within 0.2 of each other.

The Lack of Fit test can test whether significant terms are missing in the model. The strategy is to compare the amount of random variation in the residuals from the data used to fit the model with an estimate of the random variation in the process using independent data. This estimate is provided in the CCD design by replicating the centre-point and possibly other points. If the estimate of random variation obtained from the residuals is larger than the model-independent estimate, significant terms are probably missing and a significant p-value is obtained for the Lack of Fit test (Montgomery, 1995b).

Chapter 8

EXPERIMENTAL REACTION PROCEDURES

8.1 Continuous subcritical water mediated hydrolysis of natural polymer

8.1.1 Materials

Rice bran was provided by Euryza GmbH (Hamburg, Germany). Prior to the hydrolysis experiments, the rice bran was milled and sieved to recover a particle fraction of less than 180 μm . The milled and sieved rice bran was then pelleted to a mean diameter of 0.50 mm by using a drum agglomerator (Eirich, Hardheim, Germany). The reagents used for analysis were of analytical grade, ethyl alcohol and sodium hydroxide (Fisher Scientific, UK), phenolphthalein (Hopkin and Williams Ltd. Essex, UK) and diethyl ether (Carl Roth GmbH, Karlsruhe, Germany). Carbon dioxide liquid withdrawal (5 MPa @15 °C) was used in the experiments from (BOC, UK).

8.1.2 Experimental

The main part of this experimental setup is the oven, which can work up to a temperature of 400°C, having two temperatures set up panel; one is to maintain the temperature inside the column and other for the outlet valve temperature. Figures 8.1 and 8.2 show the laboratory experimental set-up and the schematic sketch respectively. Inside the oven there is a column of capacity range from 10-50 ml, to hold the desired residence time by adjusting the flow rate of HPLC feed pump. The pressure required to ensure that water remained in the liquid phase can be adjusted by feed pump, which has a capacity to work up to 40 MPa. The CO₂ can also be feed from the storage tank to the column through another HPLC pump.

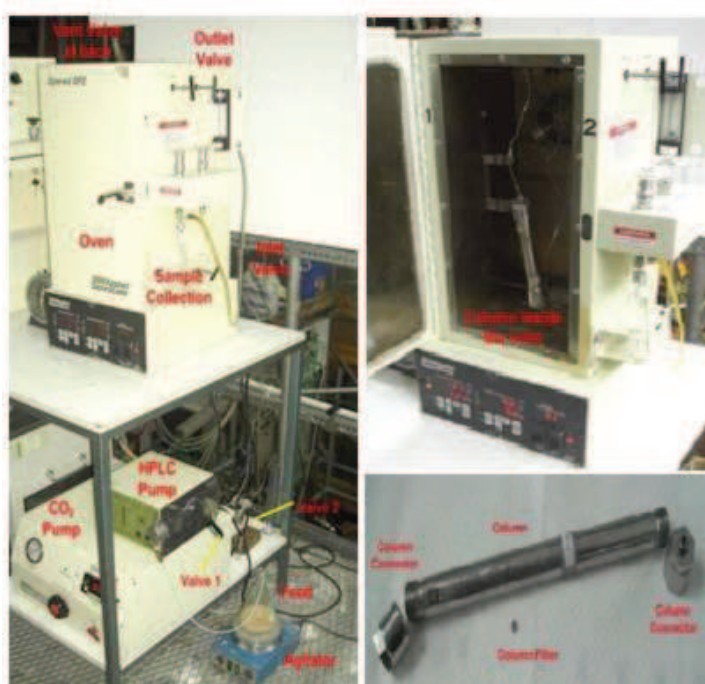


Figure 8.1 Laboratory experimental set-up of continuous extractor equipment (CEE)

8.1.3 Experimental procedure

To treat the sample without addition of CO_2 , the valve 2 and vent valve should be closed and valve 1, inlet and outlet valves open. The oven temperature is set to 200°C and the valve temperature to 60°C . Water is circulated. When the column temperature has achieved to the desired temperature, after a few minutes, the feed is allowed to circulate by switching the valve to feed side, keeping the residence time constant which can be done by adjusting the flow rate of pump. The pressure inside the column can also be maintained through the feed pump, by adjusting the throttling valve

To treat the sample with CO_2 , the feed pump is stopped, valve 1 closed and valve 2 opened. This treatment of feed has been done for a short time by closing the outlet valve. Once the treatment process has been completed, sample is collected, and the apparatus is cleaned by passing distilled water.

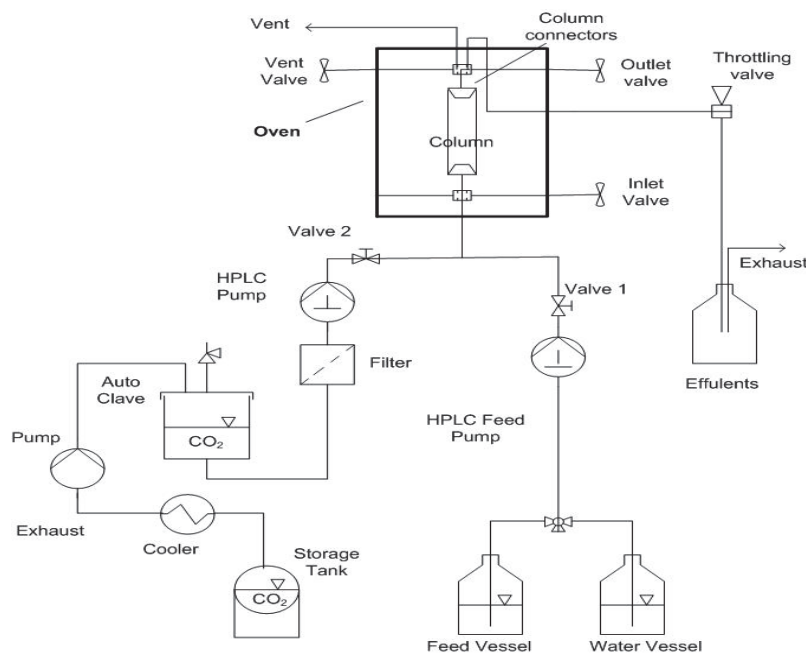


Figure 8.2 Schematic sketch of CEE

8.2 Subcritical water mediated hydrolysis of sunflower oil

8.2.1 Batch hydrolysis of sunflower oil

Batch subcritical water hydrolysis of vegetable oils was conducted using a 200 mL reactor made of Teflon which in turn was placed in the well of autoclave which was kept at a constant temperature. The effect of oil: water ratio at 1:4 and 1:2.3 were studied at a fixed temperature of 200°C and at the vapour pressure generated by the reaction temperature. The samples were analysed initially starting at the time it took to reach 200°C (~ 30 min) and thereafter at 30 min interval for a total duration of 120 min. The stirrer speed was held at 300 r/min, as to use higher

agitation rates produced a vortex, which prevented the thermocouple making contact with the solution phase.

8.2.2 Continuous hydrolysis of sunflower oil

Materials- Gases and reagents: The gases used throughout the present study were supplied by the British Oxygen Company (BOC) UK, as follows:- helium, purity 99.99%.- hydrogen, purity 99.95%. The chemicals used are listed below in Table 8.1. Distilled water was used in the experimental work to prevent scaling on the walls of the tubular reactor and ancillary piping from salt deposition under the reaction conditions. All the chemicals used in the experiments were of reagent grade or higher and were used as received, without any purification. Deionised water was used to prepare all the chemical solutions.

Table 8.1 Chemicals used for continuous subcritical water hydrolysis of sunflower oil

Material	Cas number	Grade	Supplier
Ethanol (99.9%)	64-17-5	A.R	Fisher
Sodium hydroxide	1310-73-2	A.R	Fisher
Phenolphthalein	77-09-8	A.R	Hopkin & Williams Ltd (UK)
Sunflower oil	8001-21-6	A.R	leading supermarket chain (U.K)
Hexamethyldisilazane	999-97-3	A.R	Sigma-Aldrich
Palmitic acid (99%)	57-10-3	G.C.	Sigma-Aldrich
Stearic acid	57-11-4	G.C.	Sigma-Aldrich
Oleic acid (99%)	112-80-1	G.C.	Sigma-Aldrich
Linoleic acid (99%)	60-33-3	G.C.	Sigma-Aldrich
Heptadecanoic acid (98%)	506-12-7	G.C.	Sigma-Aldrich

The sunflower oil was obtained from a leading supermarket chain in the UK and supplied in gallon batches. Although no specific composition was given for each batch of sunflower oil, the average composition analysed by GC was 97% triacylglycerols; 2.0% diacylglycerols; 0.5% monoacylglycerols; 0.3 to 0.5% FFA. This fatty acid distribution and the molecular weight together with formula from literature can be seen in Table 8.2 (Berrios et al., 2007). The short formula is expressed in C_x:y where x is the number of carbon atoms and y is the number of double bounds.

Table 8.2 Fatty acid composition of sunflower oil

Name	Short	% W/W	Formula	M. wt.	M.P. [°C]
Palmitic acid	C16:0	7.2	CH ₃ (CH ₂) ₁₄ COOH	256.43	62.9
Stearic acid	C18:0	2.7	CH ₃ (CH ₂) ₁₆ COOH	284.48	70.1
Oleic acid	C18:1	27.4	C ₁₇ H ₃₃ COOH	282.47	14.0
Linoleic acid	C18:2	61.2	C ₁₇ H ₃₁ COOH	280.46	-5.0

Experimental equipment

The hydrolysis of sunflower oil was carried out in a tubular reactor which had a total reactor volume of 60 ml. The equipment used is listed below:

- Two HPLC pumps: type 350, Gilson
- Dynamic mixer: type 811C, Gilson
- Muffle furnace: size 3, Gallenkamp, maximum 1100°C
- A reactor: stainless steel tubing of 0.45 cm i.d and 400 cm length, resulting in a volume of about 60 mL
- Coiling unit: Grant instrument Cambridge, type LTD 20G
- Butterfly valves: stainless steel Swagelok
- Needle valve : stainless steel Hoke
- Pressure gauges with transmitter: Druk, maximum 70 MPa
- Thermocouple: 12 way selection unit, K-type
- Glass feed column: Pyrex with volume of 250 ml
- Stainless steel pipe connections: 6.0 mm o.d. and 1.5 mm with thickness of 1 mm and 0.25 mm, thus giving i.d. of 4 and 1mm, respectively.
- Plastic hoses: flexible plastic hoses with 3 mm o.d/
- Gas Chromatography: GC (HP 6890) with film ionization detector (FID)
- Burette: volume of 25 ml made from Pyrex, B-grade
- Heating element: Lab-line plaza, made from Lab-Line Instrument.
- Balances: Adventure-Ohus AR-2140 (accuracy 0.0001 g) and two decimal, Metter-Toledo (accuracy 0.01g)
- Rheometer: viscosity meter (TA-1000)
- Densitometer: Anton Paar (DMA-35N). Range: 0-1,999 g/l, temperature: 0-40 °C
- pH Meter: Mettler Toledo (GMBH 8603), range: -2 -19.99, temperature: -30 +130 °C, accuracy : ± 0.01

Experimental procedure

Figure 8.3 shows the laboratory scale set-up of the equipment used for the sub critical water hydrolysis of sunflower oil under continuous flow conditions. In this system, sunflower oil and distilled water were fed separately by two HPLC pumps from the reservoir columns as shown in Figure 8.4. The mixture was then passed through an up-stream static mixer in order to mix the oil and water prior to entering the tubular reactor coil. The dosage pumps were adjusted by push

bottom switches on the pumps to give the desired volumetric water oil ratios (Figure 8.3). As the reaction conditions were not in the supercritical water region, the reactor was made from stainless steel 316 tubing. The tubular coil reactor was housed in an electrical furnace as shown in Figure 8.5. The pressure in the reactor was controlled by a back pressure regulator valve, in this way the temperature and the pressure inside the reactor coil were continuously maintained at the desired operational conditions. The reaction time t (min), was calculated by dividing the volume of the reactor V (ml) by the total volumetric flow rate of the oil and water at the experimental conditions using equation 8.1 (Minami and Saka, 2006).

$$t = \frac{V}{F_w \left(\frac{\rho_w}{\rho_w'} \right) + F_o \left(\frac{\rho_o}{\rho_o'} \right)} \quad \text{Equation 8-1}$$

where F_w is the set flow-rate of water (ml/min). ρ_w and ρ_w' are the densities (g/ml) of the water at the normal ambient and reaction conditions, respectively. F_o is the set flow-rate of oil (ml/min) and ρ_o and ρ_o' are the densities (g/ml) of the sunflower oil at ambient and reaction conditions, respectively and are assumed to be equal at the reaction conditions (Minami and Saka, 2006). F_w and F_o were measured at ambient conditions. The reactor was brought to the desired working temperature by initially pumping water through it while heating the furnace. Once the reactor reached a steady temperature, the oil pump was switched on to give the required water oil ratio. The Reynolds number as shown in equation 8.2 was calculated to be between 100 and 150 and so a plug flow system was assumed; however, the purpose of this work is not to establish a reactor equation but to deliver an optimal yield of FFA and a kinetic expression for the hydrolysis of sunflower oil.

$$R_e = \frac{\rho v D}{\mu} \quad \text{Equation 8-2}$$

Where ρ and μ are average density and viscosity respectively.

Upon leaving the reactor, the hydrolysed oil water mixture was cooled to 20°C using water bath (Fischer-UK). 5 ml of samples were then collected at required time intervals according to the experimental parameters. The hydrolysis products as shown in Figure 8.6 were gravity-separated into two fractions, the upper fraction was thought to be mainly FFA, unreacted TAG, intermediate compounds diacylglycerol (DAG) and monoacylglycerol (MAG), and the lower fraction, glycerol (glycerol with water). In this study, only the composition of the upper lipid layer was analysed by the two methods, titration and GC as explained above to determine the total yield of FFA.

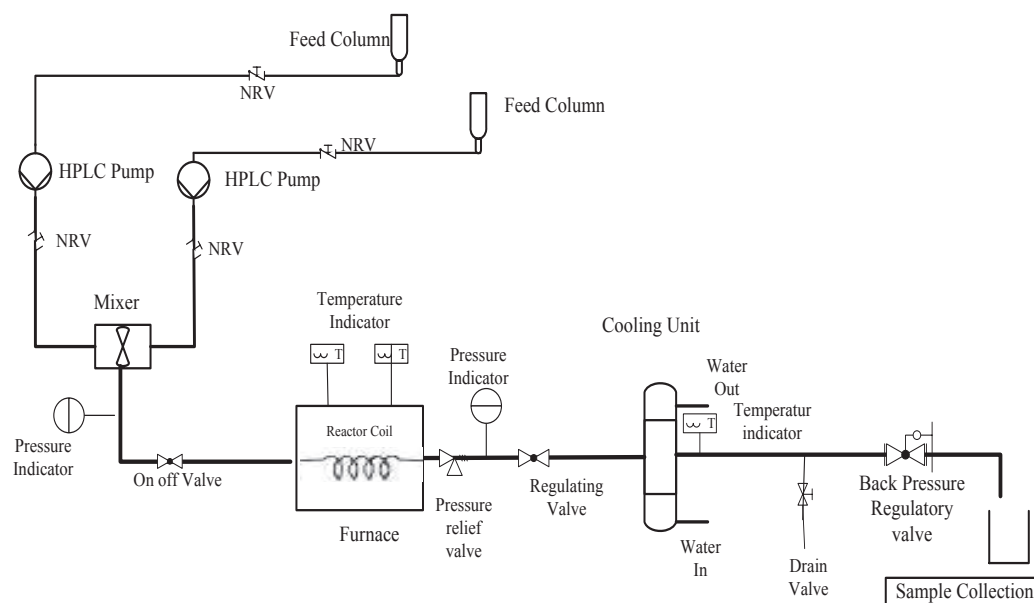


Figure 8.3 Sub-critical water continuous flow hydrolysis rig



Figure 8.4 Picture of two HPLC pump, a static mixer and feeding columns

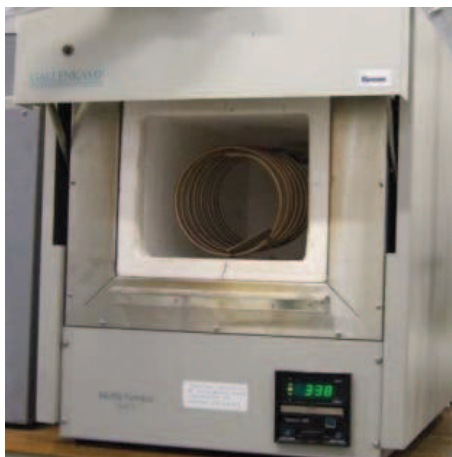


Figure 8.5 Tubular coil reactor in house of electric furnace



Figure 8.6 Sample of hydrolysed sunflower oil: upper layer (FFA, TAG, DAG, MAG) and lower layer (glycerol, water)

8.3 Lipase mediated esterification of oleic acid with ethanol

The esterification experiments were conducted with 89% pure oleic acid (Sigma Aldrich) and an immobilised lipase, Lipozyme TL IM® (Novozyme) from *Thermomyces lanuginosus* with specific activity of 250 IUN/g, on granulated silica support. Particle size was measured as 250 to 1000 μm . Bulk density was 420 kg/cm^3 and true density was 1830 kg/m^3 . Carbon dioxide (99.99%) was purchased from BOC (UK). The reagents used were ethyl alcohol (99.99%), sodium hydroxide (98.0%) and hexane (99.99%) from Fisher Scientific.

8.3.1 Enzymatic esterification of FFAs in the batch process

Three reaction media with enzyme concentrations of 5, 10 and 15wt% relative to 50 mM of oleic acid were prepared. These were each combined with 100 mM of ethanol and 15ml of hexane in a 50ml conical flask and placed in a shaking water bath (GFL-Germany) at a rotation rate of 300 rpm and temperature of 55 °C. Samples of each were taken after 2, 4, 6 and 24 hours while

agitating. The solution with a 10 wt% of enzyme was additionally studied for the reaction on addition of CO_2 .

8.3.2 Enzymatic esterification of FFAs in continuous flow process

Continuous enzymatic esterification was carried out in a packed bed reactor (TharSFC, USA) with glass beads, loaded with a given weight of lipase, Lipozyme TL IM® (Novozyme), through which a mixture of oleic acid, ethanol and SCCO_2 was pumped (Figure 8.7) at different process conditions. Mixer M1 used to ensure that the solvent and the substrate are completely mixed. All reactions were carried out in a 100 ml reactor with a working temperature of up to 200°C , pressure of 30 Mpa and loaded with enzyme. CO_2 was pumped via P3 into the reactor as shown in Figure 8.8. A sample collection vessel was used at the exit of the reactor. A backpressure regulator was used to maintain pressure in the system, and an internal heating system used to reduce the likelihood of CO_2 sublimation.

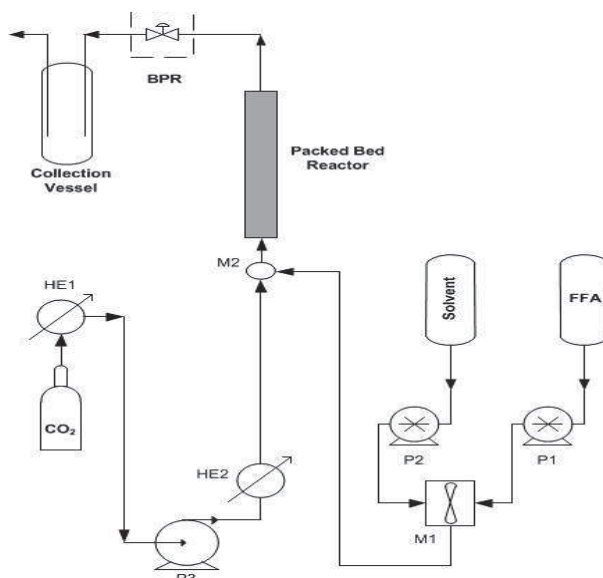


Figure 8.7 Schematic diagram of continuous flow esterification process



Figure 8.8 Continuous flow esterification process

PART C: RESULTS

Introduction

Due to the complexity of biomass and to its solid form, there is a need for a sustainable and environmentally-friendly pre-treatment process technology enabling any biomass to be fully utilised without creating associated waste streams. Critical fluids have been recognised as environmentally-benign, efficient ‘green’ solvents due to their unique physical and chemical properties, especially sub-critical water as a versatile medium for conducting both extraction and reactions (Savage, 1999). Water is cheap, non-toxic, non-combustible, and in fact it is the most benign alternative to organic solvents. Sub-critical water is just pressurized hot water, maintained below 374°C (critical temperature) and 22 MPa (critical pressure).

As detailed in Part A, sub-critical water has successfully been used as a solvent for the extraction of numerous compounds (Lanças, 2003), owing to (i) the variation of its dielectric constant according to pressure and temperature and (ii) the ion product constant which is three orders of magnitude larger for subcritical water (10^{-11}) than that of ambient water (Clifford, 1998; Savage, 1999). The acidic nature of sub-critical water provides suitable conditions for hydrolysis. Therefore given the complex interplay between extraction (solubility) and hydrolytic reactions, and the emerging potential of sub-critical water as a versatile environmentally-benign solvent, our objective was to evaluate continuous flow sub-critical water processing of feedstocks representing biomass components.

Therefore Part C gathers the results in four chapters. As said in the General Introduction, the work was oriented to allow checking the possibility of performing continuous flow biorefining of biomass, from deconstruction of structural polymers to hydrolysis of an important extractible fraction (oil), and down to functionalisation as specialty product.

Chapter 9 addresses the hydrolysis of rice bran, along with the determination of the associated kinetics.

Hydrolysis of sunflower oil is considered in Chapter 10, and enzyme catalysed synthesis of fatty acid ethyl ester is in Chapter 11.

Chapter 12 deals with the solubility of natural compounds in sub-critical water, an important parameter when dealing with heterogeneous media.

Chapter 9

SUBCRITICAL WATER MEDIATED HYDROLYSIS OF RICE BRAN AS AN EXAMPLE OF LIGNOCELLULOSIC POLYMERS

Introduction

Due to the fact biomass is in the main solid and highly complex, there is a need for an efficient, sustainable and environmentally-friendly pre-treatment process technology that will enable any biomass to be fully utilised without creating the waste streams associated with current processes. Critical fluids have been recognised as environmentally-benign, efficient ‘green’ solvents due to their unique physical and chemical properties. In particular subcritical water has attracted considerable interest as a versatile medium for supporting not only extraction of natural components but also catalytic and synthetic reactions. Water is cheap, non-toxic, non-combustible and in fact it may be the most benign alternative to organic solvents depending upon the temperature range over which it is applied and the thermal ability of the solutes being extracted or reacted. Subcritical water is pressurised liquid hot water, maintained below its critical point (374 °C critical temperature and 22 MPa critical pressure). As detailed in Chapter 4, subcritical water has successfully been used as a solvent for the extraction of numerous compounds, owing to (i) the modulation of the dielectric constant of water according to pressure and temperature and (ii) the ion product constant which is three orders of magnitude larger for subcritical water (10^{-11}) than that exhibited by ambient water. The acidic nature of subcritical water provides suitable conditions for hydrolysis. Therefore given the complex interplay between extraction (solubility) and hydrolytic reactions, and the emerging potential of sub-critical water as a versatile environmentally-benign solvent, our research objectives were to evaluate and model batch and continuous flow sub-critical water hydrolysis of lipids and carbohydrates known to account for a significant proportion of a wide range of biomass types. Therefore this chapter addresses the hydrolysis of rice bran, along with the determination of the resultant hydrolysis kinetics, while oil substrates will be considered in the next chapter.

9.1 Background of sub-critical water hydrolysis of rice bran as lignocellulosic material

Rice bran is a lignocellulosic material and the major carbohydrates in commercial rice bran are cellulose, hemicelluloses, and starch and range from 7.7 to 13.1%, 9.6 to 12.8%, 8.7 to 11.4% and 5 to 15% respectively (Saunders 1985). Cellulose consists of glucose units, linked by β -(1 \rightarrow 4) - glycosidic bonds which results in a linear polymer that supports the formation of strong intra and inter molecule hydrogen bonds. Cellulose has a high degree of crystallinity, which makes it insoluble in water, however under suitable subcritical water conditions it is rapidly hydrolysed into soluble low molecular weight oligomers. According to the reaction scheme proposed by (Dinjus and Kruse 2004), cellulose decomposes to water-soluble products, hydrolysis being the primary step. Indeed, under hydrothermal conditions, polysaccharides such as starch, cellulose and

hemicellulose, undergo hydrolysis to form oligomers and monomers such as glucose and other monosaccharides. Hemicellulose is an heteropolymer composed of monosaccharides, including xylose, mannose, glucose and galactose and aromatic/phenolic compounds e.g. ferulic acid, and due to its heterogeneous structure, it is less resistant to hydrolysis than cellulose, and easily solubilised in water at temperatures around 180 °C (Bobleter, 1994).

Hydrolysis can be conducted at ambient conditions by employing biolytic enzymes (Patil et al., 1988). To investigate the impact of multiple processing parameters on the efficacy of sub-critical water mediated biomass hydrolysis, response surface methodologies (Montgomery, 1995b) were adopted and evaluated as a potential tool to assist with the optimisation and modelling of the sub-critical water hydrolysis of sunflower oil and hydrolysis of rice bran, along with the determination of the resultant hydrolysis kinetics.

9.2 Sub-critical water hydrolysis of rice bran without CO₂

In our experiments, continuous flow sub-critical water hydrolysis of rice bran was conducted with an initial mass fraction of 1 % suspended solid in distilled water. For this dilution, we assumed that the reaction kinetics were independent of the solid concentration. For the purpose of kinetic modelling the properties of pure water were assumed (Bobleter, 1994), this was in line with the work carried out by (Rogalinski et al., 2008a) who conducted similar sub-critical water hydrolysis experiments on rye straw and rye silage. The total organic carbon in rice bran hydrolysate was measured to determine the percentage of hydrolysis H[%], compared to the total carbon in the starting biomass fed to the reactor. Sub-critical water mediated hydrolysis was carried out over the temperature range of 150-210°C (Figure 9.1). The results show that the amount of the biomass hydrolysed and therefore solubilised, increased with temperature and reaction time; this can be apportioned to changes in physicochemical properties of water associated with density, e.g. the dielectric constant, dissociation and reaction constants, which are known to vary with temperature. Rice bran when hydrolysed at 150°C for 60 min resulted in 5% solubilisation of the initial biomass and is in-line with reports in the literature (Ando et al., 2000). The percentage of the biomass which was hydrolysed progressively increased with temperature e.g. at 210°C and 60 min, 35% of rice bran was hydrolysed. The work of (Sasaki et al., 2003) shows 35% solubilisation of sugar cane bagasse at 200°C for 20 min in a semi-batch reaction mode, this shows the efficacy of sub-critical water as a solvent to support hydrolysis of biomass is dependent on both time and type of biomass being hydrolysed.

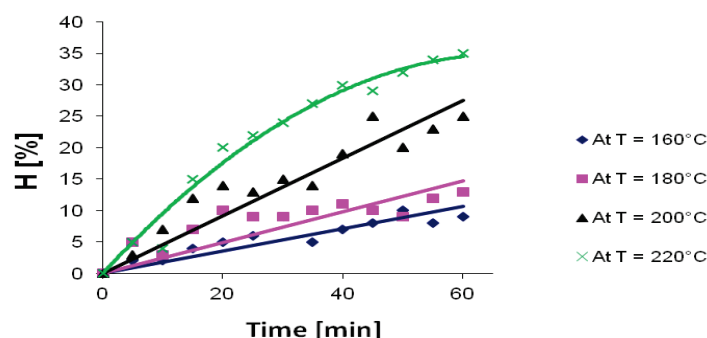


Figure 9.1 Effect of temperature on hydrolysis of rice bran in a piston reactor at 20 Mpa

A careful look at the plots, shows that the kinetics is divided in two periods. The conversion is quite fast during the first period for about 20-30 minutes then the conversion slows down while still continuing until end of plot. This is the mark of either reaction of two substrates, likely hemicellulose in the first period, but then when almost all converted, the reaction may concern cellulose, or at least hemicellulose in a more hindered position. H[%] is representing degree of liquefaction which is calculated on carbon basis ($\text{DOC}_{\text{out}} / \text{TC}_{\text{in}}$), where DOC means dissolved organic carbon and TC means total carbon. The data show that as the temperature increases so does the initial rate of hydrolysis. These results prove that sub-critical water can efficiently hydrolyse rice bran, a lignocellulose material, into a mixture of soluble carbohydrate moieties at elevated temperatures (Agbor et al., 2011).

In addition to sub-critical water mediated hydrolysis of biopolymers, it is known that the hydronium ion can act as a catalyst to accelerate the decomposition of carbohydrate monomers (Heitz et al., 1986). During the hydrolysis of carbohydrate polymers, not only are free sugars formed, but also there are well documented examples of side products such as furfural, 5-hydroxymethyl furfural (HMF), formic acid and other carboxylic acids and phenolic compounds (Kamio et al., 2006). A detailed analysis was carried to determine the amount and type of organic acids produced as an indicator of the degree of non targeted reactions occurring under the subcritical water conditions during the experiment. Figure 9.2 illustrates the results of sub-critical water mediated hydrolysis conducted at 200 °C and 20 MPa and shows the degradation of xylose obtained from rice bran into degradation products after 20 min of reaction time. A 25% yield of xylose was obtained after 40 min, its yield decreasing with time as it started to break down into furfural which was then further degraded to formic acid with extended residence time. The results are comparable to those of (Rogalinski et al., 2008a) who demonstrated that up to 10% of furfural was generated under sub-critical water mediated hydrolysis of rye straw at 120 °C in 10 min.

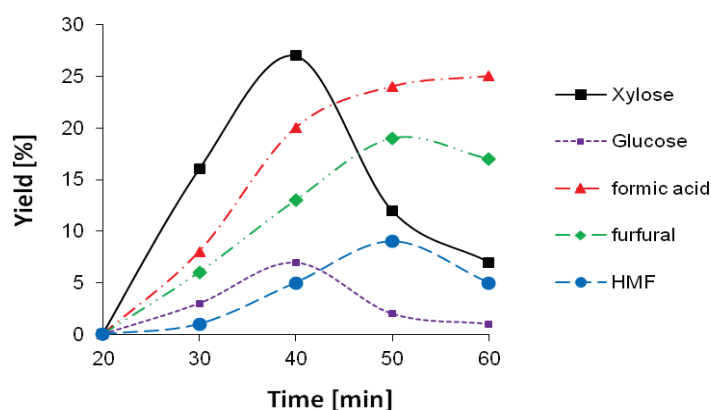


Figure 9.2 Yield of first and secondary products vs time for the hydrolysis of rice bran using sub-critical water at 20 MPa and 200°C

A detailed knowledge of products is important to optimise reaction conditions with respect to selective production of desired compounds e.g. glucose as a valuable product of the hydrolysis of lignocellulosic materials. The increased interest in sourcing glucose from cellulose rather than starch for bioethanol production reinforces the value of understanding the utility of sub-critical water to hydrolyse lignocellulose. In the present study under the sub critical water conditions used, xylose, the monomeric building block of hemicellulose, was released from the biomass in significant amounts. Although xylose represents the first principal reaction product, under extended residency times the formation of formic acid is observed and is likely a result of the protonation of xylose at the O3 hydroxyl group under acid condition. While furfural is produced by the dehydration of xylose monomers (Xing et al., 2011).

In addition, the formation of hydroxymethylfurfural (HMF) was also observed and is likely to be the result of starch hydrolysis creating glucose which is subject to dehydration through fructose to HMF (Binder and Raines, 2009).

The main objective of the studies on product formation was to find the best operating condition for hydrolysis of rice bran, and to derive practical conclusions for engineering purposes, e.g. process design and process optimisation. In the light of our results and those from the literature it is clear there is still work to be done to reduce the non-specific catalytic reaction and optimising the sub-critical mediated hydrolysis. These results shows that sub-critical water (without CO₂) can efficiently hydrolyse rice bran into a mixture of soluble carbohydrate moieties and derivatives at elevated temperatures (Agbor et al., 2011).

9.3 Influence of CO₂ addition on subcritical water mediated hydrolysis of rice bran

Currently most pretreatment processes utilise acid or alkali, as reviewed in Chapter 2. It is known that CO₂ in water forms carbonic acid, a weak acid. Therefore in order to study the impact of

carbon dioxide and thus of carbonic acid on the efficacy of sub-critical water mediated hydrolysis of rice bran, gaseous CO_2 was added to reaction medium. The goal was to determine if the CO_2 addition did indeed accelerate cleavage of the carbohydrate polymers. The influence of acidification by carbon dioxide as a function of temperature is depicted and compared to rice bran hydrolysis in pure water in Figure 9.3. The addition of carbon dioxide appears to support an enhanced rate of hydrolysis at 150 and 180°C, whereas at 210°C the same catalytic effect is not observed. A similar trend was also shown by (Rogalinski et al., 2008a) using pure cellulose as a substrate where the addition of CO_2 resulted in an enhanced rate of cellulose hydrolysis at 240°C, but the apparent catalytic effect was reduced at 260°C and 280°C. In the present case of rice bran, a complex lignocellulose material, the addition of CO_2 appears to promote enhanced hydrolysis at a lower temperature. The reduced catalytic influence of CO_2 with increasing temperature could be attributed to the following: Firstly - the drop in the pH of saturated water/ CO_2 mixtures decreases with increasing temperature, leading to a less pronounced rate of enhancement compared to the already increased rate of reaction at higher temperatures. Secondly - acidic compounds are rapidly formed in the course of reaction e.g. organic acids. Therefore the low pH values cannot be attributed directly to carbonic acid; therefore the dissociation of carbonic acid under operating conditions has a reduced impact on the pH compared to the acidic compounds formed during the reaction. Therefore auto acidification which is a function of the type of biomass could account for the impact of CO_2 on the efficacy of sub-critical water mediated hydrolysis found within the literature.

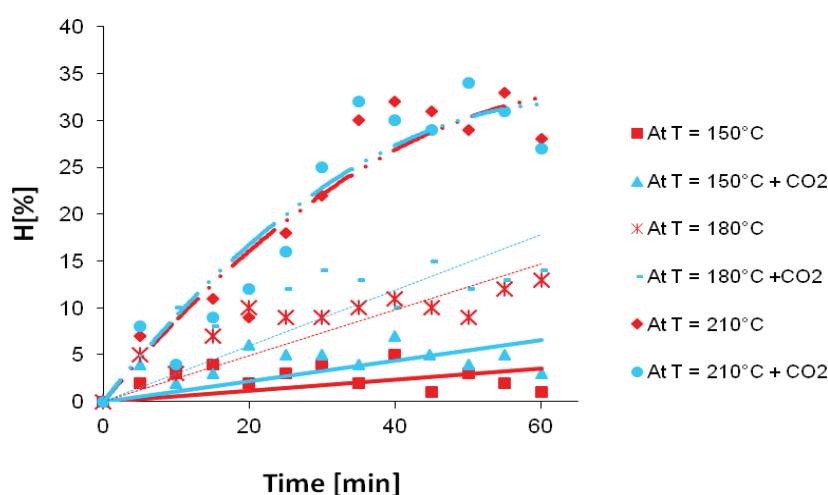


Figure 9.3 Effect of CO_2 on the sub-critical water mediated hydrolysis of rice bran vs temperature at 20 MPa

9.4 Kinetic modelling of carbohydrate hydrolysis

Some additional experiments were also performed at temperature of 150°C, 170°C, 190°C and

210°C for an accurate determination of the kinetic parameters (Figure 9.4).

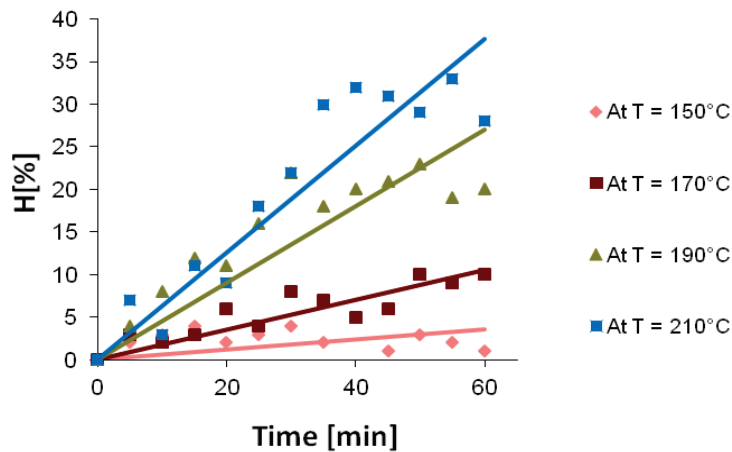


Figure 9.4 Effect of temperature on the hydrolysis of rice bran at 20 MPa

The additional data are in good agreement with the results of the previous experiments, which shows the satisfaction for the experimental set-up. The curves illustrated in Figure 9.1 and 9.4 have been used for modelling the hydrolysis conversion with a first order approach. For such an approach the degree of conversion can be written as follows:

$$L = 1 - \exp(-k \cdot \tau)$$

Equation 9-1

and after rearrangement

$$\ln(1 - L) = -k \cdot \tau$$

Equation 9-2

Where K denotes the reaction rate constant, L is the hydrolysis conversion and τ represent reaction time.

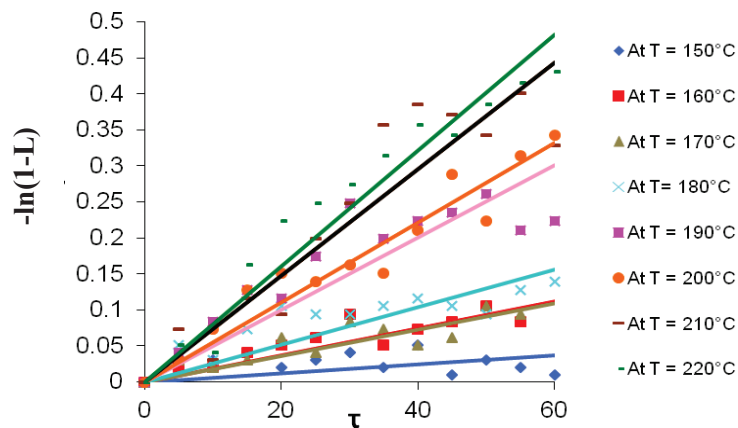


Figure 9.5 Determination of the hydrolysis average rate constant

In the case where the rate of conversion can accurately be described by a first order kinetic, the reaction rate constant can be read as the slope of $\ln(1-L)$ versus τ diagram. Such a diagram is depicted in Figure 9.5 for the temperature range of 150 to 220°C.

It can be concluded that the data are reasonably well reflected by a linear regression, although considering the kinetics as a whole, irrespective of the above mentioned two periods (two substrates). An average reaction rate constant at different temperatures can be determined by computing the slope of these averaged straight lines Table 9.1.

Table 9.1 Reaction rate constants at different temperatures

T[°C]	150	160	170	180	190	200	210	220
K(T)[min ⁻¹]	0.0166	0.0272	0.0279	0.0451	0.0753	0.0722	0.0939	0.1033

A common approach to express the temperature dependence of the reaction rate constant is the Arrhenius' law

$$K = K_o \cdot \exp\left(-\frac{E_a}{RT}\right) \quad \text{Equation 9-3}$$

In Logarithmic form Arrhenius equation is written as

$$\ln K = -\frac{E_a}{RT} + \ln K_o \quad \text{Equation 9-4}$$

Where E_a is activation energy and K_o is pre-exponential factor

Accordingly, these values can be obtained by plotting $\ln[k(T)]$ versus the reciprocal temperature and reading the ordinate intercept and the slope of the line. The Arrhenius plot with the respective values of the pre-exponential factor and the activation energy are given in Figure 9.6.

Summing up the results of this parameter study it can be concluded that the kinetics of the rice bran conversion in sub-critical water at 20 MPa, can accurately be considered by the equation stated above. From Figure 9.1 and 9.4 it can be concluded that the data can be correlated by a

linear regression fit, which suggest the reaction kinetics are first order, at least as a broad approach.

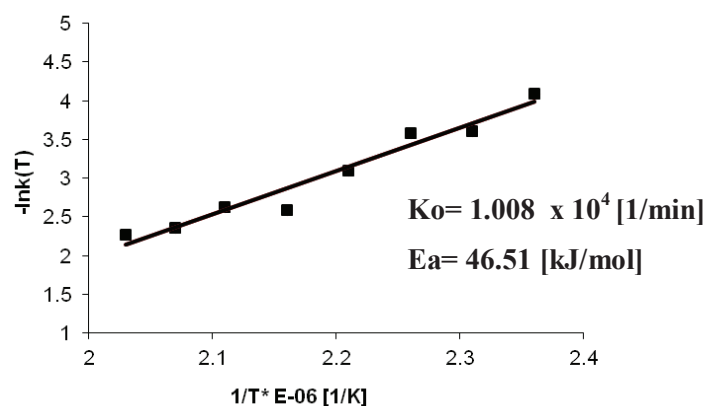


Figure 9.6 Determination of kinetic parameters from the Arrhenius plot

The experimental data was further modelled using global rate law which again confirmed that first order reaction kinetics could be applied. This global rate modelling approach is especially applicable at lower temperatures, where the data obey a linear relationship. The reaction rate constants at different temperatures can be derived by determining the values of the averaged slope of the straight lines (Table 9.1). By adopting the Arrhenius law for the reaction kinetics the pre-exponential factor (K_0) and activation energy (E_a) of the reaction can be determined, $\ln[k(T)]$ versus the reciprocal temperature and reading the ordinate intercept and the slope of the straight line (Figure 9.6).

The straight line was obtained by linear regression of the reaction rate constants at different temperatures. As can be seen, the calculated straight line is in accordance with the value of the reaction rate constants. Hence, the Arrhenius' law can be applied to the hydrolysis conversion of rice bran in sub critical water. (Rogalinski et al., 2008b) also described satisfactorily, the hydrolysis kinetics of biopolymers in sub-critical water by a global first order rate law.

The kinetic modelling for the hydrolysis of rice bran in the sub-critical water/ CO_2 system is based on pure water, and no carbon dioxide concentration dependence was assumed. As indicated in Figure 9.7, the reaction rate constants for rice bran hydrolysis in the presence of CO_2 are slightly higher than those for pure water at temperatures of 150°C to 210°C . The lines in Figure 9.7 corresponding to Arrhenius based-kinetics approach one another with increasing temperature, so that no further enhancement in the rate of liquefaction could be obtained by adding carbon dioxide at temperatures higher than 220°C . The addition of carbon dioxide however into the sub-critical water increases the liquefaction yield at the same temperatures.

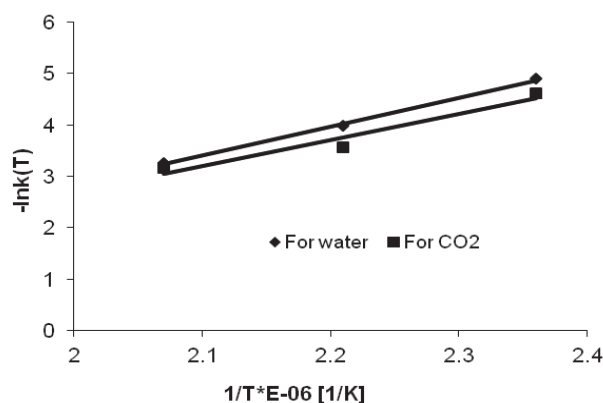


Figure 9.7 Arrhenius plot for rice bran hydrolysis with water and water/CO₂ mixtures

In summary from empirical results combined with kinetic modelling it can be concluded that the kinetics of lignocellulosic conversion in sub-critical water can be described by a global first order rate law and corroborates the work of (Rogalinski et al., 2008b). This makes sense because water is readily provided to those reaction sites in biomass which are accessible.

To accurately determine the kinetic parameters, preheating time needs to be as short as possible to fulfil an isothermal condition. In a case of non-isothermal condition, heat transfer limitations resulting from reactor configurations need to be considered when developing a kinetic model for biomass saccharification during pretreatment (Wyman and Jacobsen, 2001). The existing kinetic models provide a useful tool for predicting the rate of reaction of rice bran for cellulose and hemicelluloses during subcritical water pretreatment under various conditions. However, more progress is needed to make these models more reliable and effective by taking into account the effects of the interactions among lignin and the two carbohydrates.

9.5 Conclusion

The model substrate chosen for lignocellulosic biomass, rice bran, undergo almost complete hydrolysis of its hemicellulose component, in a rather short time (20-30 minutes) under these subcritical water conditions (~200°C, 20 MPa). Then further hydrolysis occurs at a slower rate. Addition of CO₂ induces a marked positive effect, supporting the use of lower temperature. This is the result of peculiar solvent properties of sub-critical water (ion product and dielectric constant, viscosity). This was observed with a continuous experimental set-up suggesting it could easily be apply at scale for industrial purpose. Before reaching this point further analytical work is required to monitor accurately the effect of all above tested parameters not only on the total conversion of rice bran, but also on the product selectivity between first and second products, i.e. allowing to optimise these complex reaction systems and then target high conversion towards selected products.

**RSM MODELLING OF SUBCRITICAL WATER
MEDIATED HYDROLYSIS OF SUNFLOWER OIL**

Introduction

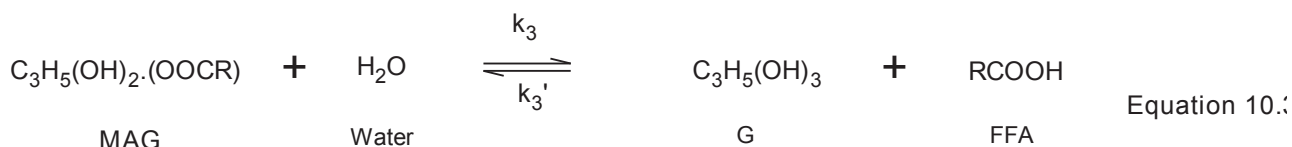
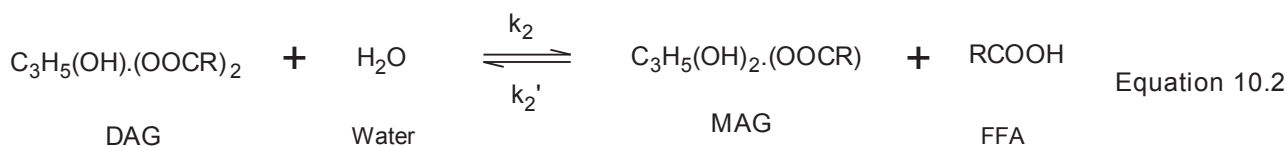
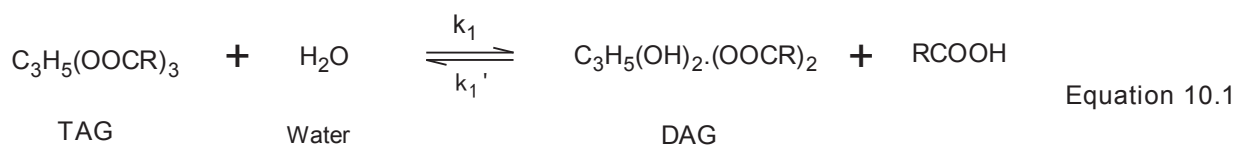
The hydrolysis reactions are commonly carried out in a batch configuration system, however, the continuous flow system was proven to work in an effective way for industrial production of FFA from vegetable oils, called fat splitting. But in this last case, the reaction is carried out under experimental conditions (250°C, 5 MPa) still far from critical point (374°C, 21.8 MPa), for practical reasons, therefore not benefiting of all useful expected advantages, although in a continuously fed reactor, and the kinetics is lower.

Most of the papers oriented towards the subcritical water hydrolysis of TAG do not go into detailed investigation of the influence of processing parameters and did not investigate deeply the reaction products, or operated under batch conditions. Therefore there was a lack of information about this apparently very simple reaction, eg hydrolysis of oil under subcritical water conditions.

This chapter discusses the results of experiments performed to study the production of FFA in the continuous flow tubular reactor. The reactions studied were simply the hydrolysis of triacylglycerols of sunflower oil with distilled water. The main objective of this work was to investigate the effect of various process variables, i.e. pressure (10-20 MPa), temperature (270-350°C), residence time (5 - 30 minutes) and the initial volume ratio of water to sunflower oil (50:50 and 80:20). The primary objective was to approach optimal conditions for obtaining the highest yield of FFA. This has been achieved through response surface methodologies (RSM) used to assist in the modelling the subcritical water mediated hydrolysis of sunflower oil. Using the empirical data, RSM models were created and successfully validated. The primary product free fatty acids (FFA) were targeted in priority, as a commodity oleochemical having a very large and well established market. Therefore the analytical work was also oriented to achieve this goal, and a GC based method and a titration method, were investigated. The former provided an insight to the evolution of the chemical composition of the reaction medium, and thus to the complex chemistry underlying the quite simple triacylglycerol hydrolysis steps. The PhD work will not detail this area, but above results will be discussed to help understanding the influence of the main process parameters in the chemical and physicochemical area, and show the high potential interest for application, of the peculiar advantages brought by the subcritical water conditions.

10.1 Background of triacylglycerol hydrolysis reaction under sub-critical water

The hydrolysis reaction has been shown by (Mills and McClain, 1949) to occur as three stepwise reactions (Equations 10.1-10.3).



According to the general classification by (March, 1982), this reaction proceeds through an AAC2 mechanism, implying an acyl cleavage. Under usual experimental conditions it is a pseudo-homogenous first order reversible reaction in the oily phase in excess of water (Ackelsberg, 1958b; Asakuma et al., 2009). We have considered all ester links be hydrolysed at same rate whatever the FA and the position of the ester on the glycerol backbone. In the first case, this assumption can be justified given the long distance and large number of C-C bonds and degree of freedom between the ester C-O-C group and the C=C double bonds, while we introduced the second hypothesis to simplify the chemical analysis work and computation, thus accepting to access to an average value for DAG and MAG. Having said this, in the first step TAG is hydrolysed to DAG, in the second step DAG is hydrolysed to MAG and in the third step MAG is hydrolysed to glycerol (G); with each step, a FFA is generated. The process for hydrolysing oil using water can be defined as a mass transfer controlled chemical reaction whereby water reacts with oil (triacylglyceride) to form free fatty acids (FFA) and glycerol (Lascaray, 1949). (Patil et al., 1988) have presented a three step reaction model which describes the thermal hydrolysis of different vegetable oils (peanut, tallow and coconut). Quantitative information on the reaction kinetics of non-catalytic vegetable oil hydrolysis is limited (Fujii et al., 2006; Minami and Saka, 2006); moreover, the kinetic data reported are not fully established as only one rate constant was considered for all the forward reactions.

The whole reaction medium was sampled at reactor outlet, and allowed to cool, conditions under which phase separation occurs as shown in Figure 8.6. The upper phase is supposed to contain all hydrolysis products (FFA, acylglycerols and any secondary products containing a long carbon chain), except glycerol, staying with water in the lower phase. Product collected FFA product is

the sum of three times the moles of reacted TAG (main constituent of starting oil), two times the moles of DAG present initially in oil reacted and the moles of initial MAG, thus equivalent to the total moles of water consumed.

(Chuang and Johannsen, 2009; King, 2012) have investigated the shift of the $\text{CO}_2 + \text{H}_2\text{O}$ equilibrium towards carbonic acid, and found that pH drops very quickly from 7 to 4, while CO_2 pressure increases of a few bars from atmospheric conditions. Then the decrease becomes linear from about pH 3 at 5 MPa, but do not reach 2.5 under 20 MPa. This effect is similar at 200°C regarding the first part of the curve, but less marked with about pH 3.5 at 20 MPa. Thus the main catalytic effect from CO_2 doped water may be affected without an expansive contribution of pressure.

In addition, FFA act as an acid catalyst for the hydrolysis reaction in sub-critical water and yields up to 90 %wt conversion without the use of any catalyst. This autocatalytic reaction of course eliminated the task of catalyst removal from the final product, being technically difficult this step would increase the cost of the final product (Demirbaş, 2003).

Under batch conditions, (Holliday et al., 1997) reported 97% of TAG conversion in 15-20 minutes at 280°C . The same team (King et al., 1997) then obtained similar results in a flow-through-reactor for shorter residence times 10-15 minutes; 12-15 MPa), but at temperature of 340°C , thus close to the critical value. In fact, in spite of existing literature about ester hydrolysis under SCW, still much less than the papers dealing with the synthesis like methanolysis, published works deal with the hydrolysis of simple monesters like methyl and propyl laurates (Khuwijitjaru et al., 2004) or even water effect during glycerolysis of TAG (Temelli et al., 1996). Most of the papers oriented towards the SCW hydrolysis of TAG, often do not go into a detailed investigation of the influence of processing parameters (Tavakoli and Yoshida, 2006) or focused on the consumption of the starting TAG and did not investigate deeply the reaction products, or operated under batch conditions (Kocsisová et al., 2006; Minami and Saka, 2006; Pinto and Lanças, 2006).

Therefore, to our knowledge, there was a lack of information about this apparently very simple reaction, e.g. hydrolysis of oil under subcritical water conditions.

10.2 Comparison between GC and titration methods for FFA analysis

The major fatty acid in the sunflower oil, as identified by GC, are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2); these contribute to 97% of the total FA content, while C18:3 makes less than 1%, as also found by (Sonntag, 1979). While the titration provides only the total acids in the upper phase supposed to measure thus the hydrolysis level, the analysis by GC can provide additional information about further reactions progressing with the

newly formed FFA. This method, detailed in Chapter 6, was therefore checked after TMS derivatization of all -OH containing compounds, to shorten the analysis time.

The comparison of concentration distribution of fatty acids measured by the silylation (TMS) / GC and titration methods is shown in Figure 10.1. Both methods compare well and give a strong correlation ($R^2 = 0.98$), though the GC tends to give higher values in particular in the 30 to 80% range. In fact while the conversion progresses there are several hypothesis that can explain some discrepancy between the two methods. For example the titration method accounts for all acid groups present in the analysed phase, thus may include dimer acids for example. At the opposite, the formed FFA may undergo condensation reactions like cyclisation to lactone, removing one carboxylic acid group. However, silylation/GC method enables a more detailed view of the reaction medium. Therefore both techniques present advantages and drawbacks. The acid value of the crude reaction product for example 91.5 corresponds well to a sample containing 92% FFA determined by GC.

We considered both methods to be acceptable for analysing the organic phase products following the sub-critical water hydrolysis.

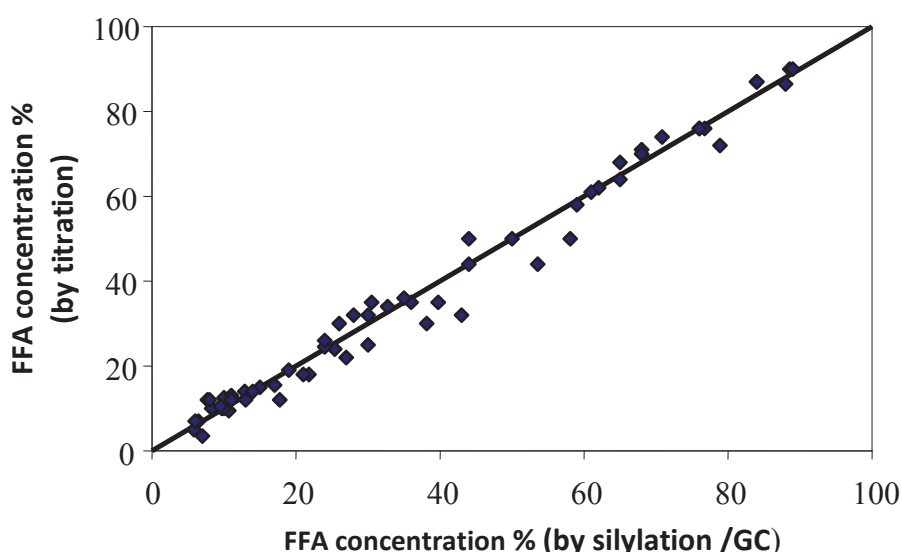


Figure 10.1 Comparison of total FFA concentration obtained by titration and GC

10.3 Evolution of the FFA composition in hydrolysed products

The starting oil is composed of 7.2% of C16:0, 2.7 % of C18:0, 27.4 % of C18:1 and 61.2 % of C18:2 (Table 10.1), these contributed to more than 97% of the total FA content, as also found by (Sonntag, 1979). The four main FA in the starting oil were obtained for example at 300°C, 15 MPa and 50:50 water/oil ratio are shown in Figure 10.2. As expected there was an increase in total FFA

yield as the reaction time progressed; the average standard deviation is in the range of 0.6 to 3.7% which is acceptable (Table 10.2). The C18:2 remained the dominant FFA after hydrolysis reactions, as in the starting oil. This result is in accordance with the percentage of FFA in the sunflower oil before hydrolysis took place as shown in Table 10.1. The percentage of each FFA is relatively constant at beginning of the hydrolysis reaction. However, depending on reaction conditions, C18:2 may be as low as 40 % or even as 30%. This may be due to the TAG profile, which is known to place preferably C18:2 in the SN2 position (central position) of glycerol backbone (Palla et al., 2012; Reske et al., 1997). Thus, under the reasonable hypothesis of higher steric hindrance on the central position of glycerol towards the attack of water molecules, one expects the production of C18:2 maximized after having produced a substantial amount of diacylglycerols (Alenezi et al., 2009). Also it may be considered that under elevated temperature we saw another peak appearing in the chromatogram while C18:2 was going down, complementing the lost C18:2 so as the total of these two FA remained almost constant and close to 60%, i.e. as in the starting oil. Although the identification of the corresponding structure of this compound being out of the scope of this work, it may be considered to be derived from the most reactive FA. Therefore the probable hypothesis is the migration of C=C double bonds, to the conjugated and therefore more stable form of C18:2. Thermal conditions are known to favour this isomerisation, although C18:2 can be more stable than C18:3. In addition it was also noted that, when the conversion is high, for example 70% FFA, and the C18:2 at 40% or lower, the unknown FA does not make anymore the balance to the initial percentage of C18:2 in the starting oil (~61%) as shown in Table 10.1. This is the mark of further reaction of the FA, especially the one bearing the higher unsaturation i.e. two C=C bonds; this will be discussed later in the chapter.

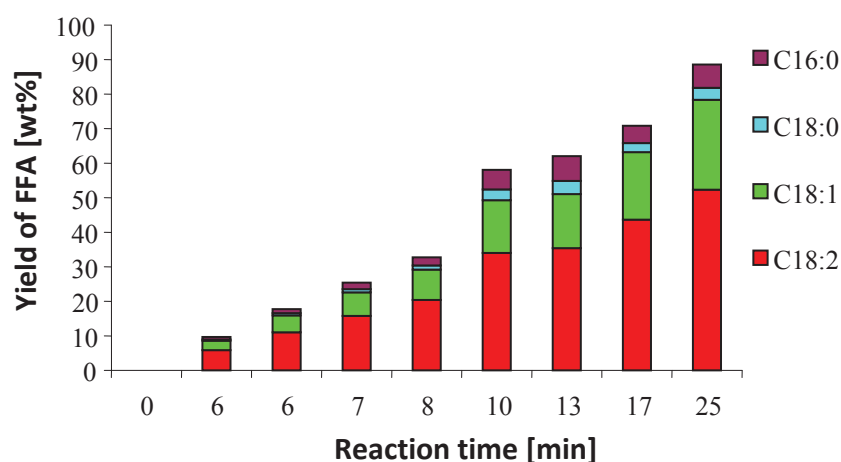


Figure 10.2 Yield of four main FFA at 15 MPa, 300°C, and water/oil ratio 50:50 v%

Table 10.1 FA composition of starting sunflower oil and in hydrolysis product

Fatty acids	Sunflower oil (%)	FFA (5.8%)	FFA (73.3%)	FFA (70.5%)
Palmitic C16:0	7.2	4.1	6.7	5.3
Stearic C18:0	2.7	3.9	4.1	5.1
Oleic C18:1	27.4	47.2	28.7	38.1
Linoleic C18:2	61.2	44.8	51.4	30.1
Unknown	0.0	0.0	9.1	21.3

Total for sunflower oil does not make 100% for accounting of the minor FA; for other columns only the listed FA were used for computing the percentage.

Table 10.2 Standard deviation for experimental data at 20 MPa and 350°C

Residence time [min]	Yield of FFA [wt%]	Standard deviation [wt%]
5.0	75.5	3.7
5.6	79.2	0.6
6.4	83.8	0.6
7.5	86.3	0.7
9.0	89.7	1.6
11.3	91.7	1.7
15.0	92.8	1.3

10.4 Effect of pressure on the FFA yield

In order to determine the effect of pressure, the hydrolysis reaction was conducted between 10 and 20 MPa while keeping the temperature constant at 300 °C and a water/oil ratio of 50:50 vol%. It can be seen from Figure 10.3 that the influence of pressure on the yield of FFA is quite small in this range. However in spite of the experimental error, it can be noted that the plots corresponding to the lower pressure (10 MPa) are always inferior to the other pressures. However, at 10 minutes of reaction time, the gap between the yield of FFA is as high as 15 %, for a FFA yield of 35 and 50% respectively at 10 and 15 MPa. After this critical gap, at 25 min of reaction time, the yields are the same within experimental error (80-85%). In the continuous flow hydrolysis of sunflower oil with sub-critical water, the optimum pressure is likely to be lower than 20 MPa. This result indicates that the degree of hydrolysis is largely independent of pressure and therefore water density under these working conditions. This effect is almost the same when changing the water:oil ratio to 80:20 instead of 50:50 as above (Figure 10.4).

10.5 Effect of temperature on FFA yield

The effect of temperature on the yield of FFA was investigated at 270, 300, 330 and 350°C at 20 MPa using 50:50 water to oil volume ratio. The effect of the reaction temperature is shown in

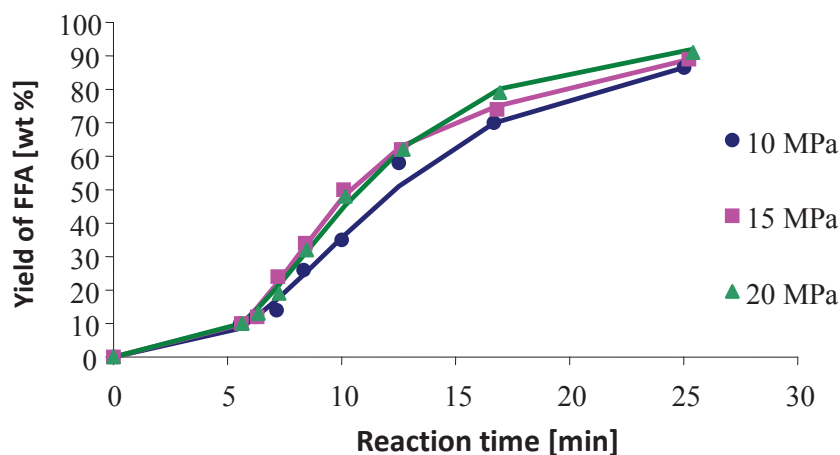


Figure 10.3 Effect of pressure on the total FFA yield at 300° C and water:oil ratio 50:50 v%.

Data points are experimental results and the lines show the best fit to the data

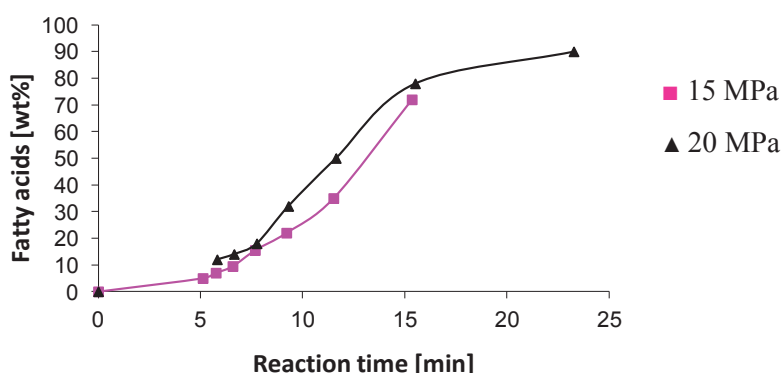


Figure 10.4 Effect of pressure in the total FFA yield at 300°C and water:oil ratio 80:20 v%.

Data points are experimental results and the lines show the best fit to the data

Figure 10.5. At 270°C the yield of FFA very slowly increases in the early stages of the reaction. It can also be noted that there is an induction period of 6-7 minutes at 270°C, while this induction time is reduced to 1 or 2 minutes if any, at 300°C and above.

As water and oil are insoluble at temperatures below 250°C, reactions under these conditions are controlled by transport properties.

At 270°C, the maximum yield of FFA was 75% at 26 min reaction time; King et al. (1999) reported that a 22% yield of FFA from soybean oil was achieved in 30 min at 270°C for a flow reactor system. This is in the same range although the present kinetics is a bit faster. Increasing the

temperature increases the oil solubility in water (Pinto and Lancas, 2006), thus reducing the duration of the induction period, as is evident here. (Ackelsberg, 1958a) reported that an increase of 10°C in the temperature increases the rate of hydrolysis by a factor of 1.2 to 1.5 while at 300°C the hydrolysis reaction reaches equilibrium around 90 wt.% of FFA after 25 min. At 330 and 350°C, the yield of FFA reached approximately 90% after 16 and 8 min reaction times respectively. However, at 330 and 350°C the yield of FFA decreased after having attained the maximum yield of 90%.

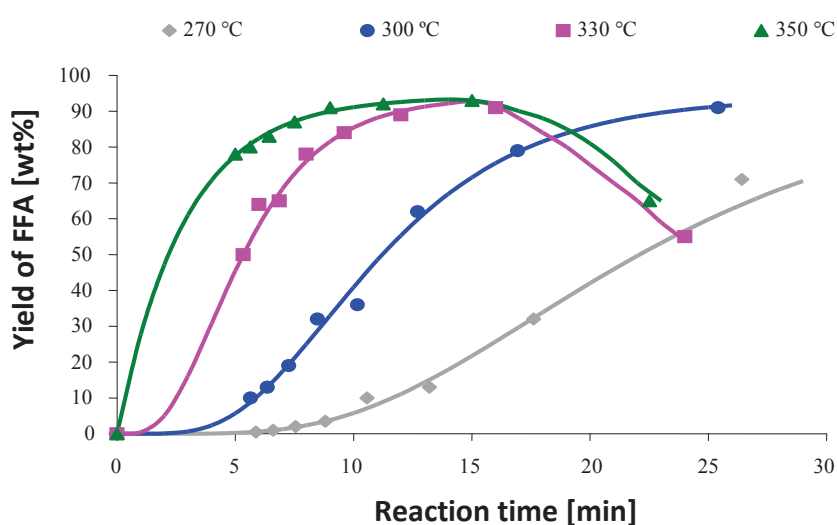


Figure 10.5 Effect of temperature on the total of FFA yield at 20 MPa and water/oil ratio 50:50 v/v%.

Data points are experimental results and the lines show the best fit to the data

(Weatherley and Rooney, 2008) have reported enhanced rates of reaction and FFA yields using a high voltage electrical field, this can be considered as a temperature effect due to localised heating. The rate of enzymatic hydrolysis of sunflower oil increased with the magnitude of the applied electrical field. These results verify that temperature has a significant influence on the reaction rate of non-catalytic hydrolysis of vegetable oil, probably linked to an increased stabilization of the widely accepted charged tetrahedral reaction intermediate. Further study performed in our team (Alenezi et al., 2009) has shown from Arrhenius plot, that the hydrolysis reaction requires greater energy to start the first reaction step, than the second and third reactions.

(Minami and Saka, 2006) observed the same induction period and a plateau around 90% of FFA, under similar conditions (continuous flow reactor; 20 MPa; water:oil volumetric ratio 1:1), and they hypothesized the effect of newly formed FFA acting as an acid catalyst in the hydrolysis

reactions. However they did not check the evolution of the FFA fraction at longer residence times after having reached this plateau. Surprisingly this plateau was reached after about 25 minutes, whatever the temperature between 290 and 320°C. This is still consistent with our observation of the marked decrease of the FFA yield after this pseudo plateau in Figure 10.5.

The drop in FFA yield with extended residence time could be for example due to polymerisation of the FFA with the unreacted MG, DG and TG or pyrolysis reactions (Holliday et al., 1997). Linfield et al., (1984) mentioned that highly unsaturated fatty acids can polymerise at temperature above 218°C.

Under present conditions, the most probable reactions are the dimerisation of FFA or even FFA and MAG owing to reasonable hypothesis of start of this reaction before complete hydrolysis of acylglycerols, or even of TAG. The dimerisation reaction is performed at industrial scale for producing glue or paint components, under thermal conditions, either with FFA or TAG respectively. The formation of dimer acids under similar conditions was proven by (Kocsisová et al., 2006), by FTIR.

Addition of the carboxylic acid to the C=C double bond could also occur, yielding lactones or estolides; in spite of these reactions being also performed in industry for fragrance or lubricant applications, it is difficult to figure whether they could also occur under sub-critical water conditions, this medium being relatively new in oleochemistry. Even in the case of fat splitting, the pressurised water conditions are far from temperature and pressure applied in our work; answering all above raised questions was out of the aim of this explorative work.

Still we would like to address the apparently surprising decrease of FFA yield already mentioned about Figure 10.5, at 300°C and above. This is the mark of consumption of FFA transformed into condensation or polymerisation products, and thus the complement of the FFA yield to 100% may be considered as a measure of the formation of these cascade reaction products. It may be concluded that they make about 60-65% of starting oil after 25 minutes at 300 and 340°C. In addition the start of these secondary reactions before having ended hydrolysis could explain why the plateau is located at about 90-92%, never reaching 100%. The error from the analytical GC method could also contribute to this gap, because of the loss of some material as glycerol in the lower phase.

10.6 Effect of water:oil ratio on FFA yield

The effect of water/oil ratio on the FFA yield at 15 MPa and 300°C is shown in Figure 10.6 A decrease in the water:oil ratio from 80:20 to 50:50 resulted in a higher FFA yield after the

induction period, between 6 and 15 minutes; then the plots are similar within experimental error. At a reaction time of 11 min, a yield of 55% FFA was obtained for a water/oil ratio of 50:50 v%, compared to 35% FFA in the dilute system when the water/oil ratio was 80:20 v%. This result is in agreement with (Lascaray, 1949) who reported that higher water ratio gave lower FFA yield in the beginning of the hydrolysis reaction but gave higher yield as the reaction proceeded. Moreover, (Sturzenegger and Sturm, 1951) also reported that FFA yield curve was not affected by water:oil ratio in the first stage of the reaction but higher water ratio gave higher final FFA yield. The rates of hydrolysis have also been reported to be higher initially at lower water to oil ratio (Desai et al., 1984).

In a way similar to the pressure effect in Figure 10.3, we observe here a critical range of reaction time or conversion, where the one parameter shows some influence. Even a strong influence because the yield of FFA doubles for reaction time situated in the middle of the critical range (about 10-12 minutes of reaction time in Figure 10.6). If looking at the “y” axis this occurs in the FFA range located between 10 and 65 % for current parameter, which is about the same range for the effect of pressure (Figure 10.3; 15 and 60 % respectively). One can note that both the ranges are very similar, and this could be the mark of a physicochemical effect due to colloidal structure (roughly emulsion properties, possibly linked to the distribution of reaction products, assumed to be similar at same FFA yield).

As a matter of fact all the products DAG, MAG, FFA are well known surfactants, even used in industrial food formulations for the formers. Therefore all these surfactants, plus non reacted TAG and water form polyphasic system, in which some of these compounds are supposed to partition according to solubility and probable intermolecular interactions. This is supposed to form a complex system, possibly polyphasic, or at least multi-micellar. Therefore we could imagine that under these peculiar conditions, there is a critical composition range of the reaction medium for which the investigated parameters (water:oil ratio, or pressure) can have a discriminating effect, regarding the contact between the reactants, namely acylglycerols and water.

Above this, following the possible acidification of the medium due to the formation of FFA and the distribution of FFA between the supposed water and oily phases or aggregates (micelles) there might be also a difference in “autocatalytic” effect. This indicates that the water/oil ratio is an essential parameter when performing catalyst-free subcritical water hydrolysis of water insoluble but liquid- phases like vegetable oils. As more FFA is produced there is a shift in the equilibrium to the right side and the reaction time decreases suggesting the need for measuring the pH of the reaction medium.

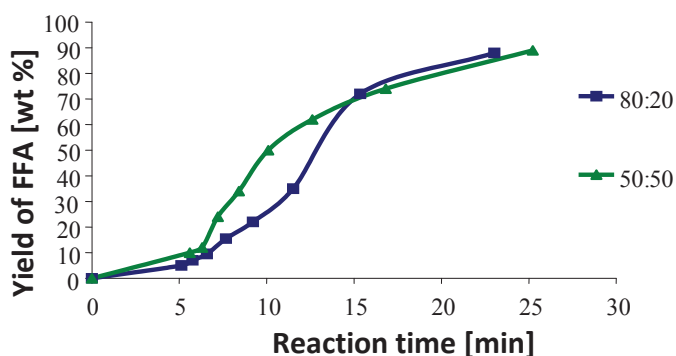


Figure 10.6 Effect of water:oil volume ratio on FFA yield at 15 MPa and 300°C

Data points are experimental results and the lines show the best fit to the data

10.7 Change of FFA yield with pH

The crude hydrolysis product corresponding to various reactions conditions, and thus FFA yields was measured for its pH. The change of pH with FFA yield can be seen in Figure 10.7. This clearly shows that when the pH is lower when the FFA yield is higher. There is a threshold value of pH or conversion. When the pH is lower than 4.0 the yield of FFA significantly quickly overpass 10%. High FFA yields induce a small but significant acidification of the medium, and this support the autocatalysis hypothesis, induced by combined effects of the presence of FFA and of the sub-critical water. There is a dramatic increase in FFA yield once the pH is lower than 3.7. Figure 10.7 indicates that at the minimum measured pH (3.2), the yield of FFA is 92%. Although talking about pH when dealing with FFA, be unusual, the complex interplay between all process parameters and formed compounds under such conditions out of the stream in organic chemistry, lead to this surprising result of substantial pH lowering effect, while starting from distilled water (pH ~7). This is in the range of the pH lowering effect of CO₂ expanded water, used in the previous chapter during this PhD work, and mentioned by (King, 2012), pH being lowered to ~3 in the case of added CO₂ while we noted here, a pH already lower than 3.5, thus falling in the same range, but without added CO₂. This practical measurement about an autocatalytic effect, often mentioned but yet not well understood, provides useful information for further industrial use. (Minami and Saka, 2006) demonstrated the autocatalytic effect, by adding 10% FFA to the oil to be hydrolysed, and this cleared the induction period.

10.8 Change of FFA yield with water density

The density is said to govern solubilisation properties of organic compounds in sub-critical water. The relationship between the water density (affected by pressure and temperature) and the yield of

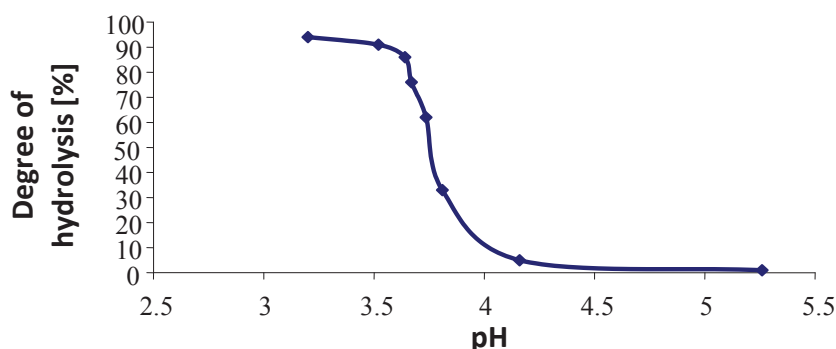


Figure 10.7 Change of pH of reaction medium vs the FFA yield

Data points are experimental results and the lines show the best fit to the data.

FFA is shown in Figure 10.8. The water density under various operating conditions (270- 350°C and 20 MPa) was obtained from the National Institute of Standards and Technology (NIST, 2009). A high yield of FFA is obtained at low water density (below 0.66 g/ml) corresponding to high temperature and “low” pressure. The yield decreases rapidly when the specific weight of water increases from 0.66 to 0.78 g/ml. This parameter therefore has the same effect on FFA yield to that of pH.

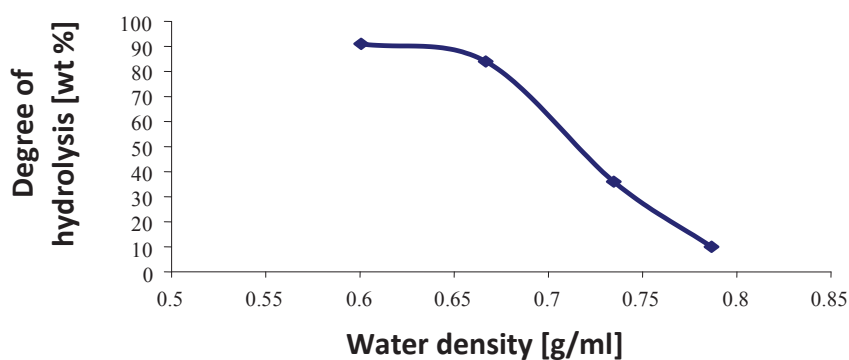


Figure 10.8 Effect of water density on the FFA yield with sunflower oil at water ratio 50:50 and 20 MPa

data points are experimental results and the lines show the best fit to the data

10.9 Optimisation of reaction conditions for continuous sunflower oil hydrolysis using RSM

In the present study, optimisation of conditions for hydrolysis of sunflower oil was carried out using the CCD (Central Composite Design). Table 10.3 lists the independent variables, the experimental design and, observed and predicted responses. The estimated response model equations 10-4 were obtained as shown below (temperature X1, °C; pressure X2, MPa; residence time X3, min; oil to water concentration X4, v/v)

$$\text{Yield(\%)} = 376.3147 - 2.7309 \times X1 - 1.0898 \times X2 - 0.4232 \times X3 + 2.0049 \times X4 + 0.0039 \times X1 \times X2 + 0.0020 \times X1 \times X3 - 0.0029 \times X1 \times X4 + 0.0007 \times X2 \times X3 - 0.0025 \times X2 \times X4 + 0.0023 \times X3 \times X4 + 0.0043 \times X1^2 + 0.0006 \times X2^2 - 0.0031 \times X3^2 - 0.0044 \times X4^2 \quad \text{Equation 10-4}$$

The results of using ANOVA for fitting the second order response surface model by the least squares method are presented in Table 10.4. The high F value (F model = 292.18) with a very low probability value (P < 0.0001) indicates the model has a significant fit. The significance of all coefficients was established by P-values shown in Table 10.4. The smaller the P value for a parameter the more significant is the impact of the experimental parameter. The linear terms; temperature, pressure and residence time, and the quadratic terms; squared reaction temperature (P-value = <0.0001) and residence time (P-value = 0.0938) made the most significant contributions to the fitted model. Furthermore, all first order interaction effects of the four predictors were statistically significant, with the greatest significance being the interaction of temperature × pressure (P-value = <0.0001) and temperature × residence time (P-value = 0.0015). At the same time, the low value of the coefficient of variation (CV = 6.19%) indicates that results of the fitted model are reliable. A high value of R² = 0.998 is an indication of high precision of the fitted model. The value of the adjusted coefficient of determination (R² Adj = 0.9951) also indicates an excellent correlation, supporting that this model explains the experimental results adequately.

Equation 10.4 was optimised to identify the optimum process conditions for maximizing the FFA yield. The process conditions predicted to yield nearly 100% FFA were: temperature 385 °C; pressure 20 MPa; residence time of 35 min; oil to water ratio 1:1.8 (v/v) represent 65% water. Experiments conducted under these conditions yielded 93.5%, FFA which is in correlation with the predicted results shown in Table 10.3.

10.10 Interactions between reaction parameters and effect on lipid hydrolysis

Three dimensional surface plots graphically represent the relationship between the responses and process parameters, and are presented in Figures 10.9-10.11. The plots were made by taking an

infinite number of combinations of the values of the two test variables at a time and keeping the values of the remaining two test variables constant. Such plots are useful for understanding the

Table 10.3 Experimental design showing actual variables studied along with the experimental and predicted yield

No	Process Variables				Yield (%)		Relative deviation (%)
	X1	X2	X3	X4	Predicted	Experimental	
1	250	20	60	80	14.49	15.28	2.67
2	310	15	35	65	19.05	18.33	1.93
3	250	10	10	50	1.48	1.65	5.54
4	250	20	10	80	4.44	4.00	5.23
5	250	10	60	50	4.52	5.41	8.95
6	310	15	35	50	14.58	15.28	2.34
7	370	20	60	50	89.47	90.71	0.69
8	310	15	60	65	23.46	22.80	1.42
9	310	15	10	65	10.66	13.40	11.38
10	370	10	60	80	49.42	50.29	0.87
11	370	15	35	65	59.53	60.63	0.92
12	310	15	35	65	19.05	18.80	0.67
13	370	20	10	50	70.43	70.03	0.28
14	310	15	35	65	19.05	18.33	1.93
15	250	15	35	65	9.59	10.11	2.66
16	370	10	10	80	30.38	30.55	0.29
17	310	20	35	65	29.56	30.32	1.27
18	310	15	35	80	21.54	22.56	2.32
19	310	15	35	65	19.05	20.92	4.67
20	310	10	35	65	11.56	12.93	5.60
21	310	15	35	65	19.05	20.45	3.54
22 ^a	385	20	35	65	99.85	93.53	3.27

^a *Validation experiment at optimal conditions*

(temperature *X1*, °C; pressure *X2*, MPa; residence time *X3*, min; oil to water concentration *X4*, v/v)

interaction of two test variables and determining their optimum levels by holding the other test variables constant.

In order to determine the effect of pressure, experiments were conducted at 10 MPa and 20 MPa while keeping the temperature constant at 310°C and the initial reactant ratio at 1:1 for oil and water. RSM models incorporating these data are shown in Figures 10.9-10.11. As a general conclusion, these indicate that at a pressure of 20 MPa with other variables at their optimum levels, the maximum yield was obtained.

In Figure 10.9 the influence of pressure and reaction temperature on sunflower oil hydrolysis is shown. At low temperatures the lipid hydrolysis increases with pressure. Minami and Saka, (2006) have shown that sub-critical water mediated hydrolysis of rapeseed oil reaches a maximum conversion of 90 %

Table 10.4 Analysis of variance ANOVA for response surface quadratic model

Source of variation	Sum of Squares	Degrees of freedom (df)	Mean Squares	F Value	p-value Prob >F
Model	10489.19	14	749.23	292.18	< 0.0001
X1-Temperature	1250.00	1	1250.00	487.47	< 0.0001
X2-Pressure	162.00	1	162.00	63.18	0.0002
X3-Res. Time	409.60	1	409.60	159.73	< 0.0001
X4-water/oil ratio	24.50	1	24.50	9.55	0.0214
X1.X2	225.63	1	225.63	87.99	< 0.0001
X1.X3	78.13	1	78.13	30.47	0.0015
X1.X4	11.03	1	11.03	4.30	0.0835
X2.X3	6.13	1	6.13	2.39	0.1732
X2.X4	5.63	1	5.63	2.19	0.1891
X3.X4	6.13	1	6.13	2.39	0.1732
X1 ²	613.83	1	613.83	239.38	< 0.0001
X2 ²	5.79	1	5.79	2.26	0.1835
X3 ²	10.15	1	10.15	3.96	0.0938
X4 ²	2.52	1	2.52	0.98	0.3598
Residual	15.39	6	2.56		
Lack of Fit	10.59	2	5.29	4.41	0.0973
Pure Error	4.80	4	1.20		
CV= 6.19%	R ² =0.9985	R ² _{Adj} =0.9951	Predicted R ² =0.7718		

at a temperature of 270°C and 20 MPa for 60 min. Under the value set for other experimental parameters (water:oil ratio 1:1.8), the pressure has also a marked effect, with no visible optimum up to 20 MPa. At the highest pressure investigated, the temperature shows an optimum at about 360°C.

Figure 10.10 shows the combined effect of pressure and residence time on the conversion to FFA. Here again, an increase in pressure causes an increase in FFA yield. The reaction time exerts a relatively weak effect. The model suggests that during the first 20 min of the reaction the rate of hydrolysis was 0.8 percent per second of the change in FFA concentration, thereafter it increased at 1.6 percent per second. The study of (Ackelsberg, 1958a) shows a temperature increase of 10°C

increases the rate of reaction by a factor of 1.2 to 1.5, similar to rate increases as a function of temperature reported by other authors.

The effect of water to oil ratio combined to pressure is shown in Figure 10.11. The conversion to FFA decreases with the ratio, at low pressure. But we note the reverse effect at higher pressure (20 MPa). Thus this parameter should be handled with caution. This may explain some effect already noted in previous paragraphs, we believe linked to the complex and combined actions of the presence and evolution of surfactants with reaction time on one side and the physicochemical structure on the other side (phase separation and/or micelles). In addition, if considering the equilibrium status of the hydrolysis-esterification reaction, an excess of water is necessary to shift the equilibrium, but the possible complex physical structure of the reaction media depending on instant conditions, an excess of water might be essential to wash glycerol out of the oil phase, allowing the reaction to go (faster) to completion as shown by (Mills and McClain, 1949). The percentage of water relative to oil usually employed is about 80%, therefore less than water:oil 1:1, corresponding to about 14 times the stoichiometric amount of water.

There are however, other factors to be considered that impose an upper limit on the water to oil ratio, as suggested by the shape of the surface response in Figure 10.11.

The position of the chemical equilibrium, in accordance with the law of mass action, will depend on the concentration of glycerol and FFA in the oil phase. Glycerol, which is practically insoluble in dry oils, becomes soluble to some extent when fats contain dissolved water. From the data published it can be seen that a fat in equilibrium with a water glycerol solution dissolves glycerol and water in such a proportion that the glycerol/water ratio is same in both the phases. Thus a lipid with 10% dissolved water in contact with a solution of 15% glycerol will contain 1.5% free glycerol in solution. The negative influence of glycerol on the progress of the hydrolysis

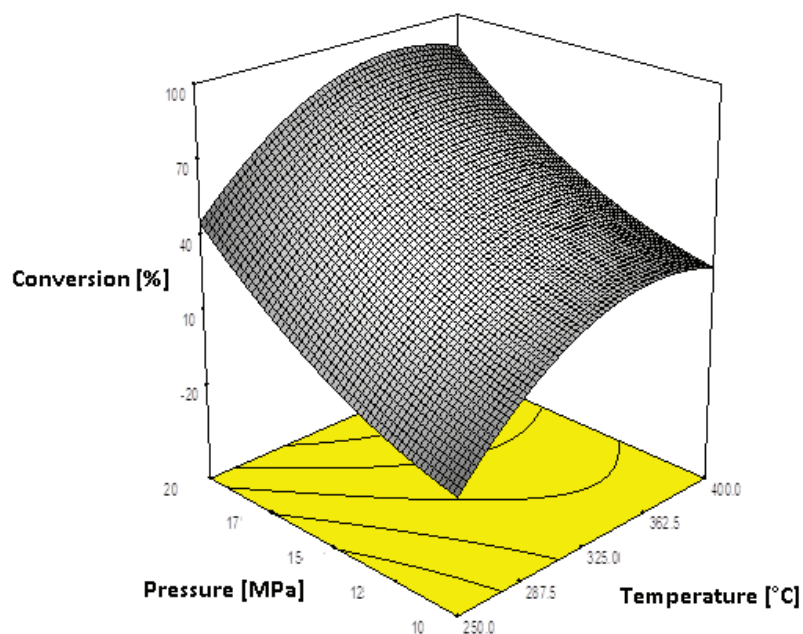


Figure 10.9 Mutual effect of temperature and pressure on percentage conversion of TAG to FFA for sunflower oil hydrolysis Residence time of 35 min and oil to water ratio of 1:1.8 v/v

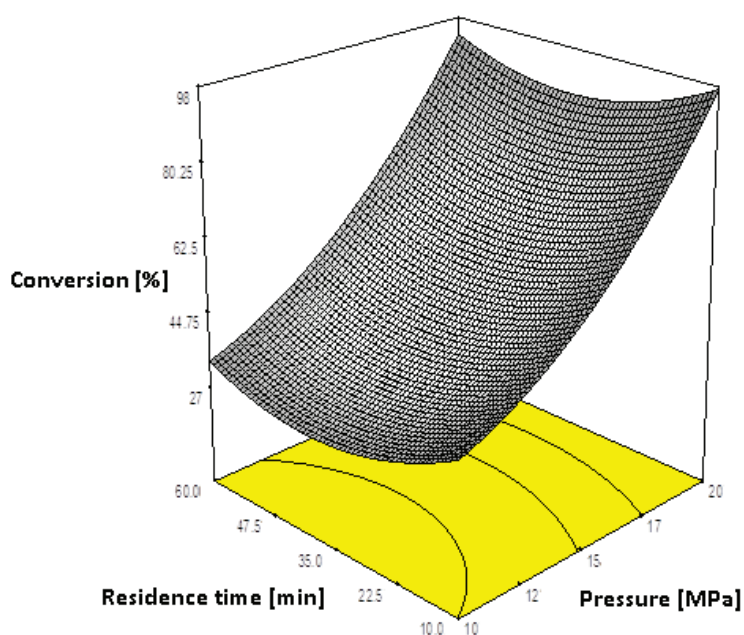


Figure 10.10 effect of pressure and residence time on percentage conversion of TAG to FFA for sunflower oil hydrolysis 330 °C and 1:1.8 v/v oil to water ratio

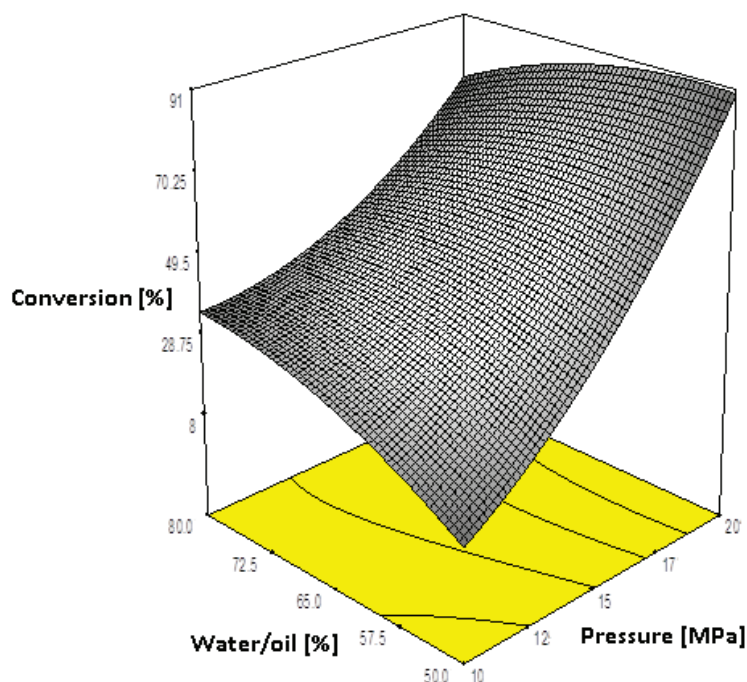


Figure 10.11 Mutual effect of pressure and oil/water ratio-in percentage on percentage conversion of TAG to FFA for sunflower oil hydrolysis Temperature 330°C and residence time of 35 min

reaction is more pronounced when the reaction approaches equilibrium. As MAG is converted to FFA, the concentration of the reacting ester decreases and the reaction slows down. The amount of free glycerol is at this stage increasing and tends to suppress further reaction. The reaction can be driven towards completion if the glycerine is removed from the oil into the water phase as quickly as possible and replaced by more water. This suggests a technical alternative, based on adding water during the course of the reaction, in the middle of the tubular reaction, as already done in fat splitting.

10.11 Properties of the produced FFA

For ending this chapter, we provide here some data relative to the physical and chemical properties of the hydrolysed product corresponding to the maximized yield of FFA that could be useful for further investigating the chemical engineering side prior to scaling up (Table 10.5). As shown there is no significant change in the density or viscosity. Since both sunflower oil and FFA are considered as Newtonian fluids, i.e. the relationship between shear stress and shear rate is linear; their viscosities do not differ by the order of magnitude (Wan, 1991). Therefore, the product and the starting oil have almost the same physical properties at inlet and outlet of the tubular reactor. The acid value determined by a standard method express the percentage of FFA, a mark of the quality of this crude product, close to technical grade (92% “purity” of FFA).

Table 10.5 Physical properties of sunflower oil and the 92% FFA reaction product

Name of component	Temperature [°C]	Density [kg/m ³]	Viscosity [Pa.s]	Acid value
Sunflower oil	25	917	5.19×10^{-2}	1
FFA (92%)	25	911	4.20×10^{-2}	183

10.12 Conclusion

The continuous process offers the advantage of large-scale production with minimum labour requirements, whilst maintaining product quality, as currently demonstrated by industrial fat splitting. The hardening of reaction conditions, in comparison to current fat splitting, allowed complete hydrolysis reactions of the starting oil (TAG) within 7 to 15 min instead of several hours, and under still industrially affordable conditions, 300-350°C, 20 MPa, and with a relatively low water consumption (low water:oil ratio); this without the addition of catalyst, even not CO₂. In spite of these hard conditions, which promote further reaction of the targeted product, high yield of 90% could be achieved. It has been found that the yield of primary hydrolysis products (FFA) is strongly affected by temperature, reaction time and the water:oil ratio; however this last parameter may have a reversed effect depending on the setting of other parameters, i.e. pressure. The continuous positive effect of pressure is more marked at low water:oil ratio, and look also to be dependent on the degree of hydrolysis. This asks for further investigating the complex interplay between the many components in the reaction medium, water, oil and the various formed surfactants. Furthermore, FFA yield is high at low pH and water density, solubility playing of course a role.

In order to achieve high yield of FFA there is a need to extend the reaction time but care should be taken to avoid the thermal degradation of the products, and it may be wise to stay around 300°C.

The autocatalytic effect, mentioned in the literature could be experienced and even measured, although the observed pH lowering effect vs TAG conversion would require further investigation, to identify the involved acidic species. Water also may act as a catalyst due to high ionic product, but also for stabilizing the supposed charged tetrahedral intermediate. The oil hydrolysis reactions exhibited an induction period which was found to decrease with an increase of the reaction temperature. This decrease, which is an improvement, can be attributed to an increased miscibility of oil and water, and to the acid lowering effect linked to autocatalysis.

The results obtained in this work provide valuable data for the optimisation of vegetable oil hydrolysis with sub-critical water in a continuous flow reactor.

The secondary products derived from produced FFA were not analysed in details and structures were not elucidated. But still it may be anticipated that these should be similar to known higher value specialty oleochemicals.

The continuous flow hydrolysis reaction of vegetable oil with sub-critical water is therefore a promising method not only for producing a high yield of FFAs, but for reaching a single step synthesis of other more complex and valuable chemicals.

**LIPASE MEDIATED ESTERIFICATION OF OLEIC ACID
WITH ETHANOL IN SCCO₂**

Introduction

Integrated biorefining is a broad all-encompassing term which describes the ambition of achieving full utilisation of primary agricultural biomass or organic waste and creating platform chemical, biofuels and materials (Foerster, 2004), and previous chapters have tempted to explore some aspects. To support this goal requires bio-processing technology development, which in turn evokes the selection of solvents. SCF are recognised as environmentally ‘green solvents’. SCF are any substances at temperatures and pressures above their thermodynamic critical point. For carbon dioxide the critical point is 7.3 MPa and 31.1°C. The temperature required to achieve critical CO₂ is compatible with mesophilic enzymes. SCCO₂ has emerged as an environmentally benign substitute for more conventional solvents and has been investigated for its suitability to support catalytic reactions (Chen et al., 2010), it has also been used extensively as a solvent for lipase catalysed reactions (Aaltonen and Rantakylä, 1991; Ballesteros et al., 1995; Kamat et al., 1995).

In light of the emerging interest in the application of SCCO₂, the objective of this chapter is to check the utility of critical fluids to support the lipase mediated transformation of FFA to ethyl esters. In the present case esterification of FFA was chosen by the industrial partner owing to (i) its availability as one of the main products of the oleochemical industry, (ii) presence of acidity in natural oleaginous feed stocks, while ethanol is currently the most abundant and cheapest marketed mono-alcohol from renewable feed stock. To achieve this objective a five factorial response surface model was designed for the continuous flow lipase mediated esterification of oleic acid with ethanol and validated.

11.1 Background about lipases in SCCO₂

SCCO₂ has already been shown to provide a stable and effective environment for synthesis and separation. However, the generally low solubility of compounds in SCCO₂ limits any kinetic advantage. If the solvent is completely removed then mass transfer limitations are likely to be more dominant, but reaction rates should improve due to higher substrate concentration (Subramaniam et al., 2002).

Biocatalysts offer significant environmental and economic benefits over conventional chemical methods of manufacture, processing due to milder reaction conditions, increased reaction efficiencies, selectivity reduces the number of process steps, and decreases use and disposal of hazardous substances (Habulin et al., 2005). The use of biocatalysts in organic solvents offers many advantages over using aqueous media. SCCO₂ exhibits properties similar to organic solvents, but with the additional capacity of transport phenomena and facilitating reaction products

separation by tuning the solvent power, thus the combination of SCCO₂ and enzymes offers significant potential with regards to developing environmentally responsible bio-processing.

Despite the existing work on lipase performance in SCCO₂ there are only a few studies which undertake the evaluation of the impact of multiple variables in a continuous flow mode relevant to process development (Turner et al., 2004). To address this issue and at that same time limit the number of empirical experiments, response surface methodology (RSM) which enables the evaluation of the effects of multiple parameters, alone or in combination, on response variables and also predicts their behaviour under a given set of conditions have been adopted (Montgomery, 1995a) and successfully validated.

11.2 Lipase mediated oleic acid esterification in batch mode

In order to understand the influence of process configuration on lipase mediated esterification a series of preliminary experiments were conducted in batch mode using hexane and supercritical CO₂ as the solvents. An immobilised lipase, Lipozyme TL IM® (Novozyme) from *Thermomyces lanuginosus* with specific activity of 250 IUN/g was selected as the preferred biocatalyst and used at varying amounts from 5 to 15%. The range of experimental parameters was chosen based on the work by (Turner et al., 2006). For the batch esterification process, three dispersions were prepared - having enzyme concentrations of 5, 10 and 15 wt% relative to 50 mM of oleic acid with 100 mM of ethanol and 15 mL of hexane in a 50 mL conical flask at 55°C. These were placed in a shaking water bath, at 300 rpm. Samples were taken after 2, 4, 6 and 24 hours. Figure 11.1 shows that under above conditions, using batch configuration, the conversion is limited to value, far from 100%, after 5-6 hours of reaction, then stays constant for one day. Due to the equilibrated character of the esterification reaction, water the second product of this reaction, staying in the reactor is expected to play against esterification. However the conversion not only the rate, but the equilibrium point, increases when increasing the load of enzyme, although not proportional. From this it looks that about 10-15 % of enzyme allow to reach the equilibrium under these given solvent and dilution conditions, which makes sense, while a much lower loading do not allow to reach the same equilibrium point, even at longer reaction times. This could be linked to some deactivation of the enzyme due to a component already present in reaction medium, or newly formed, although this observation would play in favour if the first hypothesis. Of course water is among the suspected substances; the decreased adverse effect when increasing catalyst loading could come from an adsorption or “saturation” effect happening between water, solvent and solid catalyst. This was not investigated further, but such an effect had been reported in the literature (Kamat et al., 1995).

A maximum of 40% conversion of FFA to esters can be achieved in 6 h using 15 wt % enzyme, with hexane as solvent. The CO₂ addition to the medium at 10 wt% of lipase causes a significant increase in conversion (up to 52 % in 4 h at 55 °C using 10 wt% enzyme) in comparison to hexane as a solvent which gave only 20%, under same catalyst loading, mass ratio of solvent to ethanol. This is due to the fact that ethanol and water are more soluble in SCCO₂ than in n-hexane (Bamberger et al., 1988; CHI et al., 1988). A detailed comparison of both the solvents was conducted by (Dumont et al., 1992; Marty et al., 1992) who found that the rate of reaction was influenced by the concentration of water and the composition of the reaction medium. Their findings did not conclusively show that a supercritical fluid has confirmed advantages over conventional solvents for enzymatic reactions. Our results have shown that SCCO₂ is a better medium compared to hexane, and they are supported by (Chi et al., 1988) using lipase in SCCO₂ at 50°C at 29.4 MPa in batch reactor.

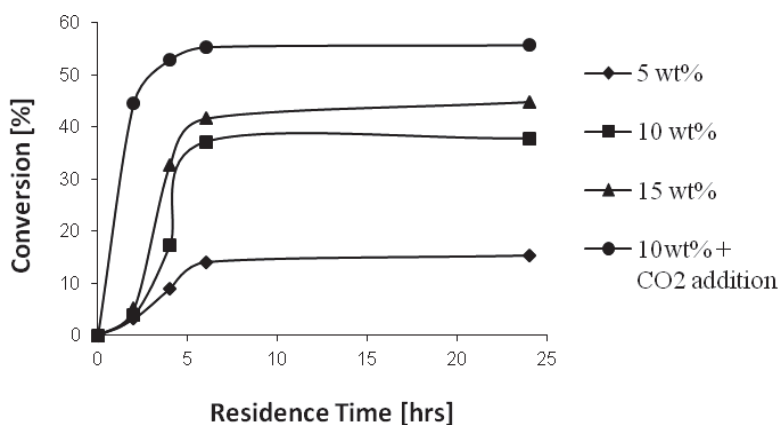


Figure 11.1 Effect of enzyme load and addition of CO₂ on the rate of esterification reaction

11.3 Experimental design

RSM based on calibrating full quadratic models around the CCD was adopted to develop a five factorial experimental design to optimise the reaction conditions for lipase mediated esterification of oleic acid under SCCO₂. Similar statistical techniques (Shekarchizadeh et al., 2009) were adopted for the enzymatic synthesis of cocoa butter analogue from camel hump fat in SCCO₂. The following experimental parameters, pressure of the reaction X1 (MPa); temperature of the reaction X2 (°C); ethanol concentration X3 (volume percent of oleic acid (v%)); enzyme concentration X4 (weight percentage of the reactant-Oleic acid) and X5, residence time, (min); were selected as the variables to maximize the response (conversion of FFA to ester). The experimental design using the RSM suggested 32 experiments with five variables at five levels (Table 11.1). A second order polynomial equation was developed to study the effects of variables on the esterification. The

equation 11-1 indicates the effect of variables in terms of linear, quadratic and cross-product terms.

$$Y = A_0 + \sum_{i=1}^N A_i X_i + \sum_{i=1}^N A_{ii} X_i^2 + \sum_{i=1}^{N-1} \sum_{j=i+1}^N A_{ij} X_i X_j \quad \text{Equation 11-1}$$

Here Y is the conversion of FFA to ester (%), X_i the variable, A_0 the constant term, A_i the coefficient of the linear terms, A_{ii} the coefficient of the quadratic terms, A_{ij} the coefficient of the cross-product terms and N the number of variables. The coefficients of the equation were determined by using Design Expert 7.0 software. The analysis of variance ANOVA and the lack-of-fit for the final predictive equation were also studied (Table 11.2). A graphical representation of the equation 11-1 in the form of 3D plots was used to describe the individual and cumulative effect of the test variables on the response.

11.4 Optimisation of reaction parameters for continuous flow fatty acid esterification

From a process development perspective the yields and associated residency time are limiting. To address this problem a test rig (Figure 8.8) was designed and made, that would support continuous flow lipase mediated esterification using SCCO_2 as the solvent.

The objective of the research was to develop a clear understanding of the impact that the operating parameters have on the efficiency of lipase mediated fatty acid esterification, in order to optimise the reaction under continuous flow SCCO_2 conditions. Table 11.1 illustrates the combination of parameters evaluated as dictated by the experimental plan generated using RSM. The empirical results deviated from the predicted rate derived from the RSM model within a range of 5% which is considered an acceptable technical error (Table 11.2). The reaction trials were measured performed in triplicate. The final estimated response model equations were obtained as below (in terms of original factors):

$$\begin{aligned} \text{Conversion } [\%] = & -205.501 + 1.375 X_1 + 3.920 X_2 + 32.910 X_3 - 5.051 X_4 - 0.189 X_5 \\ & - 0.009 X_1 \cdot X_2 - 0.193 X_1 \cdot X_3 - 0.039 X_1 \cdot X_4 - 0.002 X_1 \cdot X_5 + 0.443 X_2 \cdot X_3 \\ & + 0.103 X_2 \cdot X_4 + 0.015 X_2 \cdot X_5 + 2.025 X_3 \cdot X_4 + 0.353 X_3 \cdot X_5 + 0.073 X_4 \cdot X_5 \\ & - 0.00002 X_1^2 - 0.044 X_2^2 - 18.768 X_3^2 + 0.309 X_4^2 - 0.006 X_5^2 \end{aligned}$$

Equation 11-2

Table 11.1 Central Composite Design, experimental data for five-level five factors response surface analysis

Exp No.	Pressure [MPa] X1	Temp [°C] X2	Ethanol conc.[v%] X3	Enzyme conc. [wt%] X4	Time [min] X5	Predicted conversion [%]	Observed conversion [%]
1	15	50	1.5	7.5	40	60.2	61.5
2	20	60	1.0	5.0	60	66.2	67.0
3	20	60	2.0	10.0	60	93.5	95.0
4	25	50	1.5	7.5	40	78.1	76.5
5	15	50	1.5	7.5	40	60.2	60.0
6	20	60	1.0	10.0	20	44.1	45.0
7	10	40	2.0	10.0	60	82.9	85.0
8	15	50	1.5	7.5	40	60.2	61.0
9	10	60	1.0	5.0	20	3.1	3.5
10	15	30	1.5	7.5	40	40.3	37.5
11	20	40	1.0	10.0	60	88.1	90.0
12	15	50	1.5	2.5	40	44.1	43.5
13	15	50	1.5	7.5	40	60.2	61.5
14	20	40	1.0	5.0	20	60.6	61.0
15	15	50	0.5	7.5	40	42.4	40.0
16	15	50	1.5	7.5	40	60.2	60.0
17	15	50	1.5	7.5	40	60.2	62.0
18	10	60	1.0	10.0	60	85.1	87.0
19	10	40	2.0	5.0	20	-0.6	0.0
20	15	70	1.5	7.5	40	44.5	43.0
21	10	60	2.0	5.0	60	59.0	60.0
22	10	60	2.0	10.0	20	40.9	42.0
23	20	40	2.0	5.0	60	64.0	65.0
24	10	40	1.0	5.0	60	35.6	37.0
25	20	60	2.0	5.0	20	19.0	19.0
26	15	50	1.5	7.5	80	92.7	89.0
27	15	50	1.5	7.5	10	22.1	21.0
28	15	50	2.5	7.5	40	40.5	38.5
29	10	40	1.0	10.0	20	23.5	25.0
30	20	40	2.0	10.0	20	39.9	41.0
31	5	50	1.5	7.5	40	41.7	39.0
32	15	50	1.5	12.5	40	91.8	88.0
33*	20	60	2.0	10.9	60	100	94.3

^a Validation experiment at optimal conditions

The results of ANOVA for fitting the second order response surface model by a least square method are presented in Table 11.2. The high F value (F model = 113.85) with very low

Table 11.2 Analysis of variance (ANOVA) for the fit of the experimental data to response surface quadratic mode

Source of variation	Sum of squares	Degree of freedom [df]	Mean squares	F value	P value
Model	18946.97	20	947.35	113.86	< 0.0001
X1-Pressure	1989.26	1	1989.26	239.08	< 0.0001
X2-Temperature	27.09	1	27.09	3.26	0.0986
X3-Ethanol Conc.	5.51	1	5.51	0.66	0.4330
X4-Enzyme Conc.	3420.09	1	3420.09	411.05	< 0.0001
X5-ResidenceTime	9868.23	1	9868.23	1186.04	< 0.0001
X1.X2	365.77	1	365.77	43.96	< 0.0001
X1.X3	375.39	1	375.39	45.12	< 0.0001
X1.X4	395.02	1	395.02	47.48	< 0.0001
X1.X5	141.02	1	141.02	16.95	0.0017
X2.X3	78.77	1	78.77	9.47	0.0105
X2.X4	107.64	1	107.64	12.94	0.0042
X2.X5	153.14	1	153.14	18.41	0.0013
X3.X4	102.52	1	102.52	12.32	0.0049
X3.X5	199.52	1	199.52	23.98	0.0005
X4.X5	213.89	1	213.89	25.71	0.0004
X1²	0.13	1	0.13	0.02	0.9016
X2²	582.14	1	582.14	69.97	< 0.0001
X3²	649.51	1	649.51	78.06	< 0.0001
X4²	110.21	1	110.21	13.25	0.0039
X5²	140.73	1	140.73	16.91	0.0017
Residual	91.52	11	8.32		
Lack of Fit	88.02	6	14.67	20.96	0.0021
Pure Error	3.50	5	0.70		
Total	19038.49	31			
CV = 5.41%	R² = 0.995	R²_{Adj} = 0.986	Predicted R² = 0.883		

probability value ($P < 0.0001$) indicates the high significance of the fitted model. The goodness of fit of the model was evaluated by the adjusted coefficient of determination (R^2). The significance of all coefficients was established by P-values shown in Table 11.2. The smaller the P value for a parameter the more significant a parameter is, this reflects the relative importance of the term attached to that parameter (Khuri and Cornell, 1987). In linear terms pressure, enzyme concentration and residence time, and in quadratic terms, squared reaction temperature and solvent concentration ($P\text{-value} = < 0.0001$) made the most significant contributions to the fitted model. Furthermore, all first order interaction effects of the five predictors were statistically significant, with the greatest significance for the interaction of pressure x temperature, pressure \times solvent concentration, and pressure x enzyme concentration ($P\text{-value} = < 0.0001$). At the same time, the

low value of the coefficient of variation ($CV = 5.41\%$) indicates that results of the fitted model are reliable. A high value of ($R^2 = 0.995$) is an indication of reasonable precision of the model fitted. The value of the adjusted coefficient of determination ($R^2_{Adj} = 0.986$) is also very high, supporting the significance of the model. Figure 12.2 shows that the values of the response predicted from the empirical model are in agreement with the observed values over the selected range of the operating variables with a reasonable high value of coefficient of determination ($R^2 = 0.995$). As can be seen in figure 11.2, most of the points are located in the conversion range 50–95%. Only a couple of points are located below 50%. The residuals must be normally distributed for the results of ANOVA to be valid (Coheren and Cox, 1957). Normal probability of the residuals was checked by a normal probability plot (Figure 11.3). The plot approximates a straight line, confirming normality of the data. As the fitted model equation 11-2 provides a good approximation to the experimental results, the model was employed to find the values of the process variables for maximum yield of the fatty acid ester.

3D surfaces plots, which are the graphical representation of the relationship between the responses and process parameters, are presented in Figures 11.4 -11.8. The plots were made by taking an infinite number of combinations of the values of the two test variables at a time and keeping the values of the remaining test variables constant. The plots are useful for understanding the interaction of two test variables and determining their optimum levels while holding other test variables constant.

The response surface equation 11-2 was optimised to identify the optimum process conditions for the maximum conversion of oleic acid. The process conditions that predicted a 100% conversion were: pressure 20 MPa, temperature 60°C, ethanol concentration 2 v%, enzyme 11 wt% and residence time of 60 minutes. Experiments conducted under these conditions gave a conversion of 94.3%. The difference could be attributed to the noise generated in the model equation.

11.5 Effect of pressure on the ethyl oleate yield

The reaction pressure primarily influences enzyme activity, whereas the effect on enzyme stability is less pronounced. An increase in pressure of the supercritical fluid normally enhances the conversion rate due to the increased analyte solubility (Miller et al., 1990; Randolph et al., 1991: 219 - 237), however, at some point, the enzyme activity starts decreasing with increasing pressure (Heo et al., 2000; Overmeyer et al., 1999). This has been attributed to the lower mass transfer rates of reactants with increase in $SCCO_2$ density (Heo et al., 2000).

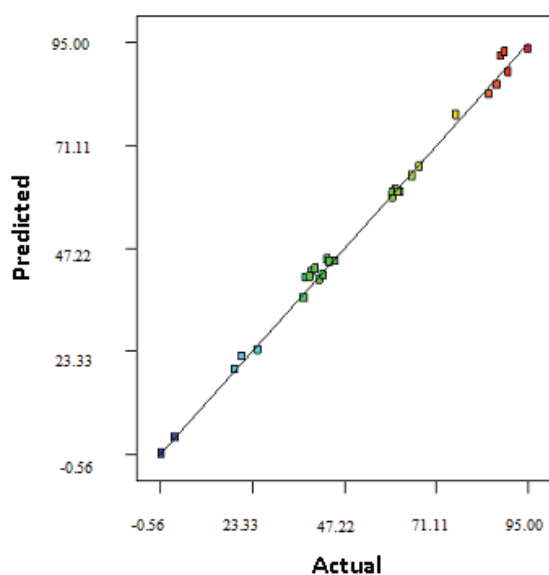


Figure 11.2 Plot of predicted response values versus the actual response values

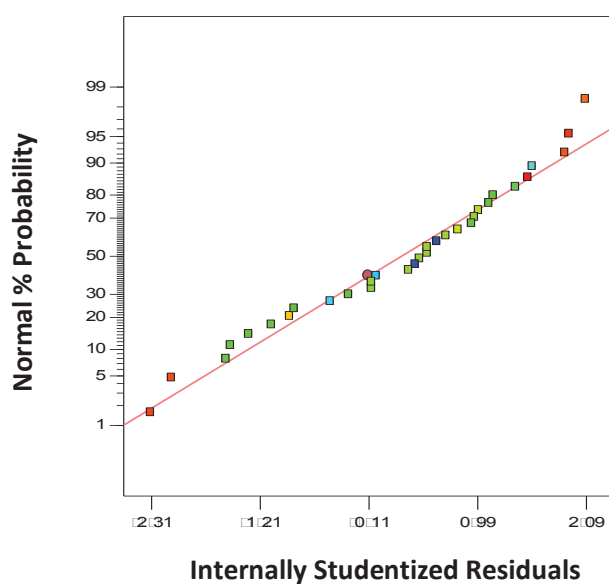


Figure: 11.3 Normal probability plot of residuals

The 3D plots Figures 11.4 to 11.8 indicate that, the maximum ester yield (70%) was obtained at the maximum pressure of 20 Mpa, of course with the other variables at their optimum levels. The pressure range of 10-20 MPa was studied previously and Knez et al., (1998) reported the pressure of 15 MPa as optimum, due to the unchanged activity of the enzyme, for the esterification of oleic acid with oleyl alcohol using *Rhizomucor miehei* lipase with SCCO_2 . A change in pressure can affect the density dependent physical properties (partition coefficient, dielectric constant and

Hildebrandt solubility parameter) of SCCO₂ which can indirectly modulate enzyme activity, specificity and stability (Kamat et al., 1993).

Figures 11.4 to 11.8 shows that the conversion didn't increased significantly with an increase in pressure, in the range studied (10-20 MPa). Figure 11.4 shows that at lower pressures at 40°C the conversion is 20%. By increasing the temperature to 60°C the conversion increases by up to 50% this effect is not pronounced at higher pressure as only 60% conversion occurs at 60°C with a pressure of 20 MPa, at higher temperatures the risk of thermally denaturing the enzyme is increased (Turner et al., 2004). Thus this effect is more pronounced at lower temperature (40°C; Figure 11.4), the conversion being doubled when doubling pressure from 10 to 20 Mpa. The same pattern is visible, in conjunction with ethanol concentration (Figure 11.6), enzyme concentration (Figure 11.7), and time (Figure 11.8). This could be because by increasing pressure, the solubility of the substrate in the SCCO₂ phase increases and decreases in the enzyme phase, with which the reactions take place (or at interface). This causes dilution of the substrate and results in the slowing of the esterification. Higher temperatures also contribute to the differential partition of the substrates in the SCCO₂ phase and the enzyme phase, along with increased pressure, as found by (Shishikura et al., 1994).

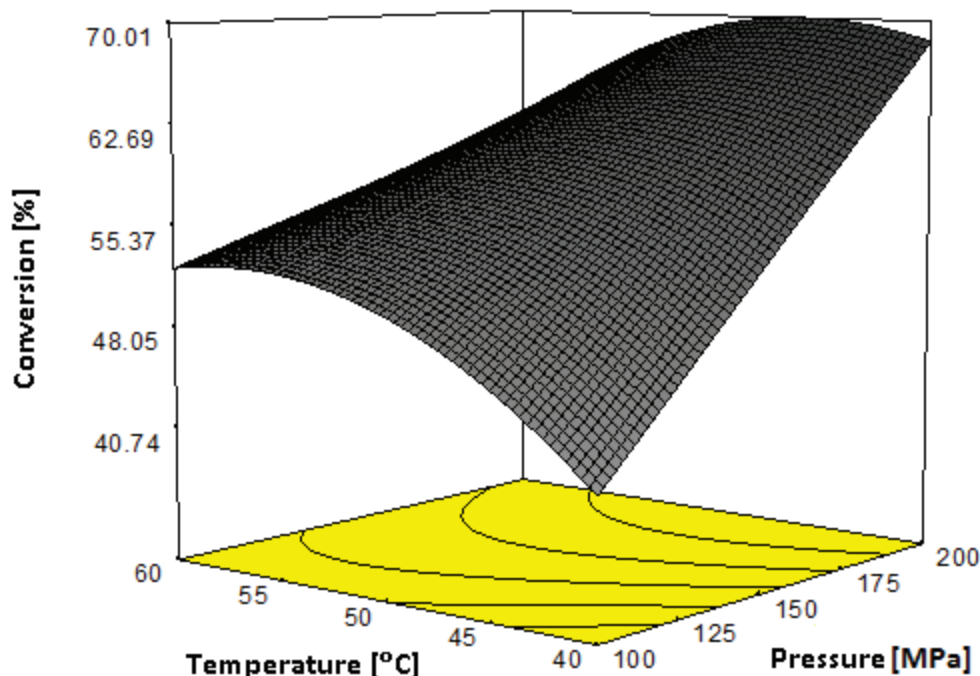


Figure: 11.4 Combined effect of temperature and pressure at 40 min, 7.50 wt% enzyme and 1.50 M ethanol concentration on the conversion of oleic acid

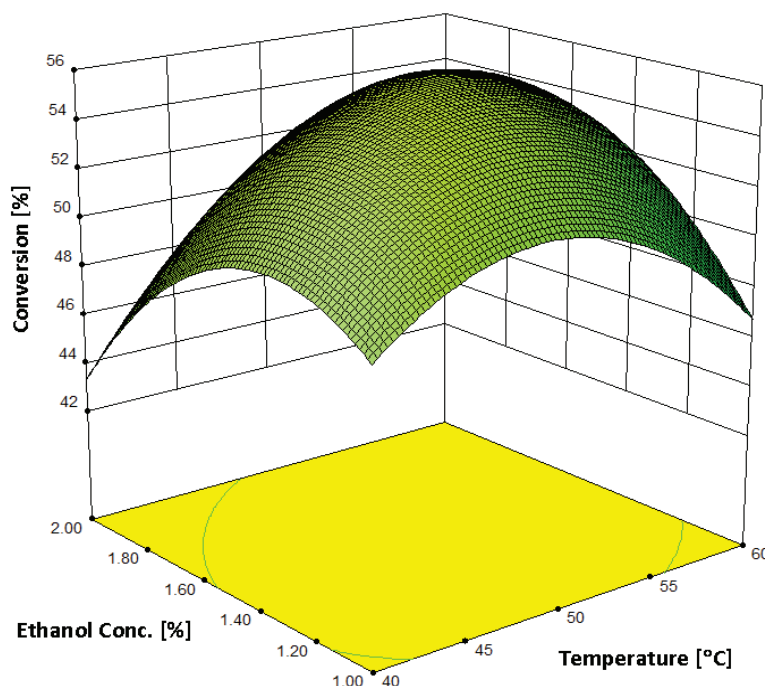


Figure 11.5 Combined effect of temperature and ethanol concentration at 40 min, 7.50 wt% enzyme and pressure of 15 MPa on the conversion of oleic acid

11.6 Effect of temperature on the ethyl oleate yield

As already noted temperature significantly affected enzyme catalysis in SCCO₂ with an optimum at 60°C (Figure 11.4). The conversion decreased at temperatures below and above this value. At 60 °C, an ethanol concentration of 2 v% gives 55% conversion as observed in Figure 11.5. The conjunction of ethanol concentration and temperature clearly shows a maximum at 60°C.

Temperature influences the 3D structure and hence stability of the enzyme. As mentioned in section 11.5 higher temperatures increase the risk of thermally denaturing the enzyme. It also affects the partition of substrates between the SCCO₂ phase and enzyme phase (Varma and Madras, 2008), and increasing temperature increases diffusion rates, thereby resulting in faster reactions, since the reaction rate is, in most cases, limited by slow diffusion of large molecules into the porous enzyme support material. Figure 11.5 shows that at higher temperatures there is a lower conversion due to the lower availability of the reactant (ethanol) on one side – also reported by Ramamurthi et al., (1991), while increased concentrations of ethanol may have the same effect. (Ergan et al., 1990; Knez et al., 1990) reported that ethanol can enhance the deactivation effect of temperature on enzyme activity. There is usually an optimal reaction temperature, which is dependent on type of enzyme, support material, immobilization technique and reaction medium, as exemplified in Figure 11.5.

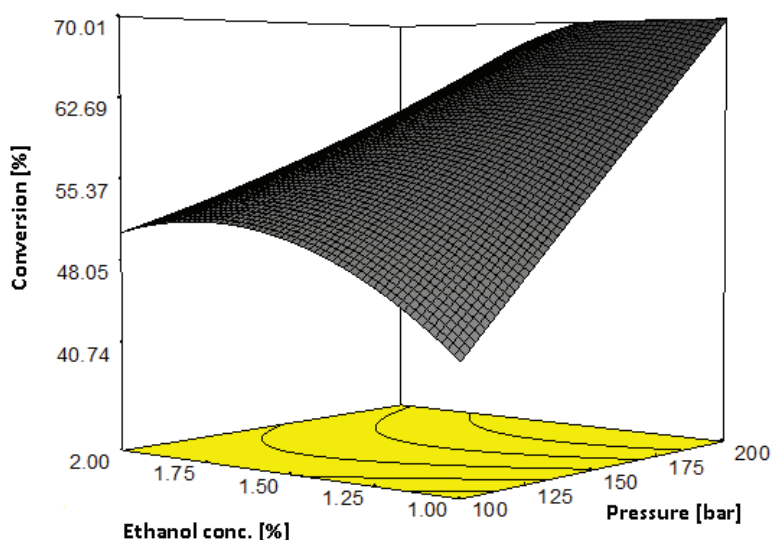


Figure: 11.6 Combined effect of ethanol concentration and pressure at 50°C, 40 min and 7.50 wt% enzyme on the conversion of oleic acid

11.7 Effect of ethanol and enzyme concentration

Figures 11.5 and 11.6 show the effect of ethanol concentration with other reaction parameters, more or less important effect on conversion, but always an optimum value. At an ethanol concentration of 2 v%, increased enzyme concentration, lower temperatures and higher residence time were found to give the maximum conversion. The decreased ethanol concentration eventually causes the incomplete conversion of the FFA into its ester. On the other hand, ethanol concentration above optimum levels was detrimental to the action of the enzyme with the interaction of other reaction parameters particularly the high temperature levels. Alcohols as substrates alone, at high concentrations have the ability to inhibit the catalytic activity of lipases (Wong et al., 2000). This optimum value is more pronounced at lower pressure.

Enzyme concentration was found to be a prominent parameter in obtaining maximum conversion (Figure 11.7). The existing results (Garcia et al., 1999) are comparable to those in this study, i.e. the effect of enzyme concentration on the conversion of FFA into ethyl ester at increased enzyme concentrations gives increased yield of ester in the range (5–15 %). The saturation effect of enzyme on the conversion was not observed within the experimental range studied. Higher concentrations of enzyme are required to achieve increased conversion at lower pressures. At high levels of pressure, 60–70 % conversion can be obtained with lower enzyme concentrations, but maximum yield cannot be achieved under the investigated range of parameters. Hence, high level of enzyme concentration (10 wt %) is necessary to obtain the maximum of ethyl ester, with all other reaction parameters at their optimum levels.

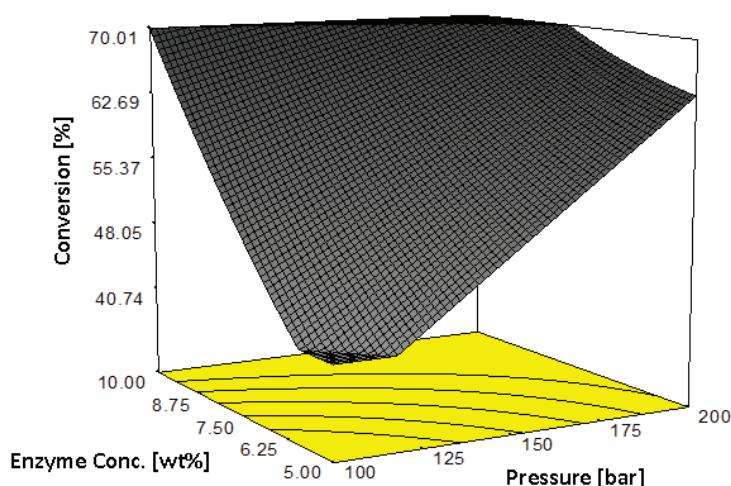


Figure: 11.7 Combined effect of enzyme concentration and pressure at 50 °C, 40 min and 1.50 ethanol concentration on the conversion of oleic acid

11.8 Effect of residence time

Talking about the combined effects of catalyst load and residence time, the 3D plot in Figure 11.8 shows that, under optimum reaction conditions, there was a steady increase in the conversion with increased residence time. But an increase in pressure does not induce any significant improvement in the conversion with increased residence time. Maximum conversion of 95% of FAEE (as shows in experimental design Table 11.1) is possible with 60 min of residence time with SCCO₂. At higher temperatures, it is possible to achieve high conversion, in less residence time, as the rate of the reaction seems to be faster, but maximum conversion cannot be obtained. In turn the increased pressure decreased the conversion. The reason for this observed effect was explained in section 11.5.

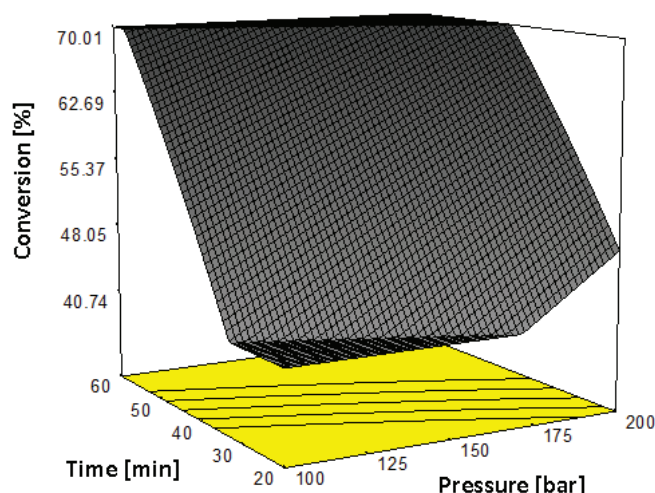


Figure: 11.8 Combined effect of time and pressure at 50°C, 7.50 wt% enzyme and 1.50 ethanol concentration on the conversion of oleic acid

11.9 Rate of synthesis of ethyl oleate per unit mass of biocatalyst

The reaction was carried out using continuous packed bed reactor (Figure 8.7 and Appendix B) with 5mg of, Lypozyme TL IM, at 100 bar, 40°C, 7.89 mM oleic acid and 42 mM of ethanol (detailed mass balance calculations in Appendix B). The aim was to determine the initial rate of reaction (or activity) per unit mass of biocatalyst by using equation 11-3, which is a form of the steady state plug flow model (Levenspiel, 1972a). It assumes that the density of the medium is constant at all conversions and that axial (forward and backward) mixing is negligible. (Alain et al., 1994) has demonstrated that the esterification of oleic acid with ethanol in a continuous packed bed reactor may be satisfactory described by the Ping-Pong Bi-Bi mechanism (Segel, 1975a). A special multi-substrate reaction in which, for a two-substrate, two-product (i.e., bi-bi) system, an enzyme reacts with one substrate to form a product and a modified enzyme, the latter then reacting with a second substrate to form a second, final product, and regenerating the original enzyme. An example of such a mechanism is found in the aminotransferases. More complex ping-pong mechanisms exist for enzymes having more than two substrates.

$$-V_{OA} = \frac{Q_{SCCO_2} C_{OAi} X_{OA}}{m} \quad \text{Equation 11-3}$$

A low substrate concentrations it was assumed that the overall volumetric flow rate throughout the reactor was that of the solvent. Data were therefore required that related solvent flow rate (Q_{SCCO_2}) with conversion, X_{OA} for a given mass of biocatalyst, m and initial oleic acid concentration, C_{OAi} . Mass balance was used to predict the required substrate flow rate for a given concentration and solvent flow rate. Such a balance was required to determine the solvent volumetric flow rate at system pressure and temperature. The oleic acid conversion was calculated using the initial oleic acid concentration and the concentration in the exit stream which comes to 6.70 mL/min giving a 15% conversion and hence the rate of reaction ($-V_{OA}$) is 1585 $\mu\text{moles/min/gcat}$.

11.10 Conclusion

All reaction variables have their own effect on the conversion of oleic acid into ethyl ester; this has been shown by CCD design under $SCCO_2$ in association with other parameters. The mutual effect of temperature and ethanol concentration on the conversion of FAEE is highly pronounced. The effect of pressure on the stability of enzymes is quite small. The predicted model fits well with the experimental results. The esterification process of FFA, as part of the biomodification process under $SCCO_2$ in a continuous configuration of reactor is found to have promising potential compared to the shake-flask method as it shows faster reaction. That is, under $SCCO_2$ esterification is achieved in 60 min to the maximum of 95%, whereas with the shake – flask method it tooks 6 h to obtain a yield of 55%.

**MODELING SOLUTE SOLUBILITY IN HIGH
TEMPERATURE WATER**

Introduction

The use of sub and supercritical fluids for the production of renewable fuels and chemicals has considerable potential; for example, hot compressed water is currently employed for fuel production from sustainable resources (Sasaki et al., 2004). Renewable sources of energy and chemicals are being sought as alternatives for petroleum derived products - the most common renewable fuel today is ethanol. The recalcitrant nature of biomass requires pretreatment to alter the structure and make the carbohydrate polymers more accessible and soluble, so that after pretreatment, the resultant monomers (glucose and xylose) can be further processed in neat solvents or with the aid of an enzyme-, microbial-, or thermally-based process. Likewise, the synthesis of biodiesel can be achieved using either SC-CO₂ (Jackson and King, 1996) or supercritical methanol (Madras et al., 2004; Saka and Kusdiana, 2001), and several research groups have demonstrated that combinations of pressurized CO₂ and water are effective in the transformation of biomass (Albrecht and Brunner, 2003; Moreschi et al., 2004; Peter van Walsum and Shi, 2004).

Sub-critical water has the potential for initially comminuting biomass followed by selective extraction of value-added chemicals. Post extraction treatment, particularly the depolymerisation of carbohydrate-based polymers via pressurized hydrolysis to monomers, for subsequent fermentation to ethanol or other chemicals, is currently being studied by our research group (King et al., 2005a; King et al., 2005b). This chapter discusses the above processes on a molecular level and to optimise processing conditions for the sub-critical water extraction and reaction of biomass and conversion of plant-derived oils to biodiesel.

Fundamental to the design and optimisation of biomass conversion processes using critical fluids is the evaluation of the solubility of substrates and products in the critical fluid medium. In addition, developing a better understanding of the conditions (state, pressure, temperature, density) for substrate and product solubilisation, and/or recovery of the resultant carbochemicals can be important for process design. Gray et al., (2003) have noted that oligomer solubility could be important in controlling the rates and yields in the thermochemical hydrolysis of carbohydrate-based biomass substrates. Similarly, knowledge of solute diffusion rates as a function of temperature in aqueous media may also impact on the rate and recovery of hydrolysates or thermochemically-generated chemicals using pressurised hot water.

12.1 Solubility data and calculations

Solute solubility in hot pressurized water above its boiling point has previously been correlated by Clifford and Hawthorne, (2002) based largely on the data generated by Hawthorne and colleagues (Miller and Hawthorne, 1999). We have expanded this database in particular by

using the compendium of Yalkowsky and He, (2003) examined the equations 12-1 to 12-3 and their applicability for predicting solute solubility in pressurized water. The Yaws equation 1, although accurate with respect to experimental data is somewhat limited as to its applicability with respect to solute type, i.e., hydrocarbon solutes. As noted by Clifford and Hawthorne, (2002), their cubic equation predicts solute solubility as a function of temperature within an order of magnitude, but serious discrepancies can begin to occur beyond the boiling point of water, therefore limiting the applicability of correlation.

Yaws Equation

$$\log S = A + \frac{B}{T(K)} + \frac{C}{T^2(K)}$$

Equation 12-1

Where S is aqueous solubility of solute and A,B and C are solute dependent parameters

Clifford Hawthorne Equation

$$\ln x_1(T) = \left(\frac{T_o}{T}\right) [x_1(T_o)] + 15 \left[\left(\frac{T}{293}\right) - 1\right]^3$$

Equation 12-2

Where x_1 is the solute solubility and T_o is reference temperature

Equation from Mathis et al.

$$\ln x_1(T) = \left(\frac{T_o}{T}\right) [x_1(T_o)] + 2 \left[\left(\frac{T - T_o}{T_o}\right) - 1\right]^3$$

Equation 12-3

Recently, del Valle et al., (2006) have proposed the following equation, Equation 12-4, for estimating the solubility of a variety of solutes in hot compressed water based on available solute solubility data, both above and below the boiling point of water:

$$\log x_2 = \log x_{2o} + (5225 - 11.75.T_c + 2431.\omega - 2.403.(T - 298.2)) \left(\frac{1}{298.2} - \frac{1}{T}\right)$$

Equation 12-4

Utilising solubility data from diverse sources, appropriate conversions and equations have been derived for converting all solubility data to a mole fraction basis, while the other required

physicochemical parameters, such as density, melting point, boiling point, critical temperature, and acentric parameter have been estimated largely by group contribution-based methods. In equation 12-4, x_2 is the mole fraction solubility at temperature, T , and x_{20} the mole fraction solubility at a reference temperature, T_0 , and x_{20} for each solute as fitting parameters. The solubility model equation 12-4 is based on the equation given by Curren and King, (2001) relating the change of solute solubility with temperature according to a van't Hoff dependence.

For all solid solutes having a melting temperature (T_m) above the test temperature (T), their corrected mole fraction solubility was estimated using the equation 12-5 proposed by Yalkowski and Banerjee, (1991) as:

$$[\ln(x_2) - 0.023026(T_m - T)], \text{ if } T_m > T$$

Equation 12-5

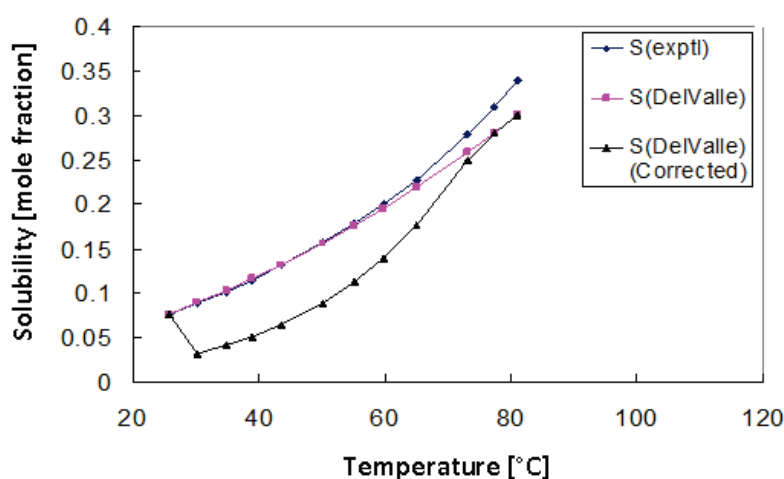


Figure 12.1 Comparison of the predicted solubility of erythritol in water with experimental data

Figure 12.1 shows a comparison between the solubility values predicted using Equation 12-4 and the experimental data, as well as the effect of the solute melting point on the corrected solute solubility mole fraction in water for erythritol.

12.2 Utilisation of the solubility parameter concept

The use of the solubility parameter to illustrate the solubility relationships of solutes in critical fluids has been demonstrated by numerous investigators (King, 1989). When combined with a reduced state correlation, the solubility parameter concept potentially provides a semi-quantitative tool to predict optimal operating parameters i.e. pressure, temperature, etc. that should be selected for efficient extractions of target solutes. The change in the solubility parameter with increasing

temperature is shown in Figure 12.2 for water, methanol, and ethanol using a Z_c (critical compressibility) of 0.23 for water and methanol, and 0.25 for ethanol. The values calculated in units of $\text{cal}^{1/2}/\text{cc}^{3/2}$ are consistent with those reported for the fluids in their condensed liquid state at room temperature. From Figure 12.2 it can be seen that the solubility parameter of ethanol, methanol and water initially decreases monotonically with increased temperature but as the respective critical point are reached the solute solubility parameter drops sharply. The solubility parameter values at the critical point reflect a substantial loss in cohesion energy density, and numerically are equivalent to those exhibited by non-polar hydrocarbon solvents. This reflects why fatty acid methanolysis is effective when performed in sub- and super-critical methanol since the fluid's solubility parameter is close to the solubility parameter of triacylglycerol based vegetable oils.

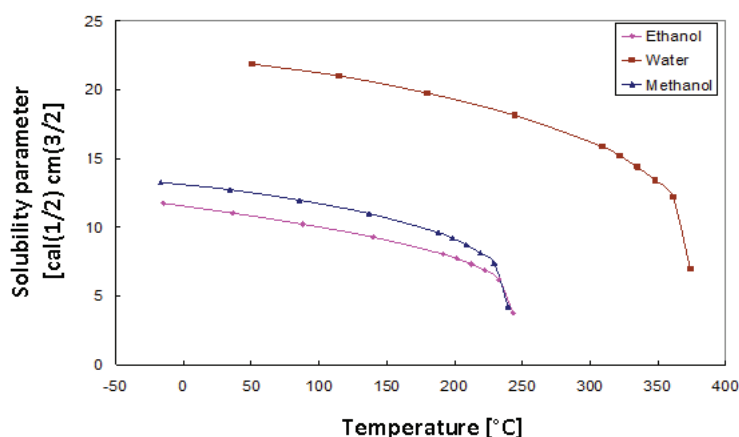


Figure 12.2 Solubility parameter variation with temperature

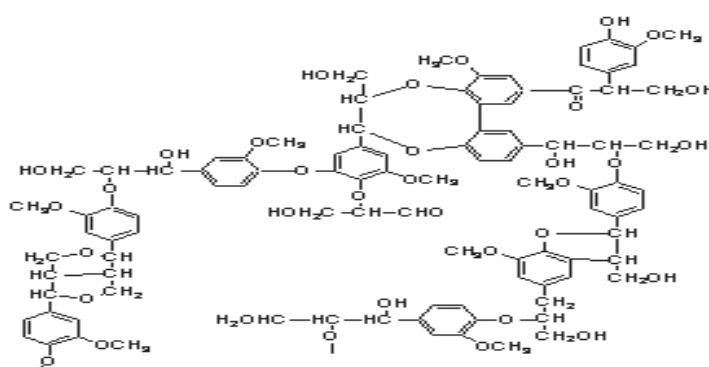


Figure 12.3 Lignin monomer molecular structure

Another advantage of the solubility parameter approach is that it can be correlated with complex molecular structures via a molecular group structural approach (Fedors, 1974). This has been demonstrated previously for supercritical fluids using simple molecules; here we have extended the

concept to model biopolymers which constitute biomass. For example in Figure 12.3 a typical lignin monomer structure is shown. Although the lignin molecule is a complex macromolecule, the group structure approach allows the cohesion energy density of the repeating oligomeric unit in the biopolymer to be calculated, permitting comparisons with those exhibited by a sub- or super-critical fluid as a function of temperature and pressure. When the solubility parameters of the fluid and solute (polymer) are equivalent, or within 2.5 Hildebrand units of each other, some degree of solubilisation will be achieved, i.e., conditions favouring extraction and reaction.

12.3 Trends and correlations for solute solubility in subcritical water

Typically solute solubility increases substantially with temperature beyond the boiling point of water, although this trend varies with solute type and may gradually level off as the solubility parameter of the solute approaches that for water at the same temperature and pressure. Solute solubility in sub-critical water can be estimated by several methods as noted in the previous section with varying degrees of success. Mole fraction solute solubility in water vary inversely with the log of their octanol-water partition coefficients (Baum, 1998); however the correlation has considerable scatter yielding a R^2 value 0.7263. Figure 12.4 shows the calculated mole fraction solubility for erythritol using the del Valle et al. correlation extrapolated from experimental data determined at lower temperatures, x_2 approaching 0.8 at a temperature of 175°C. Similar trends and x_2 values are found for monomeric and dimeric sugar solutes in sub-critical water over the same temperature range. For the training set of solutes (431 data points – 34 solutes), the del Valle et al. correlates well with the experimentally-determined solubility data ($\log x_2^{\text{exp}}$) on the log-log plot as shown in Figure 12.5. However applying this equation universally to all solutes types and classes should be done with caution and its applicability should be probed using experimental data whenever possible.

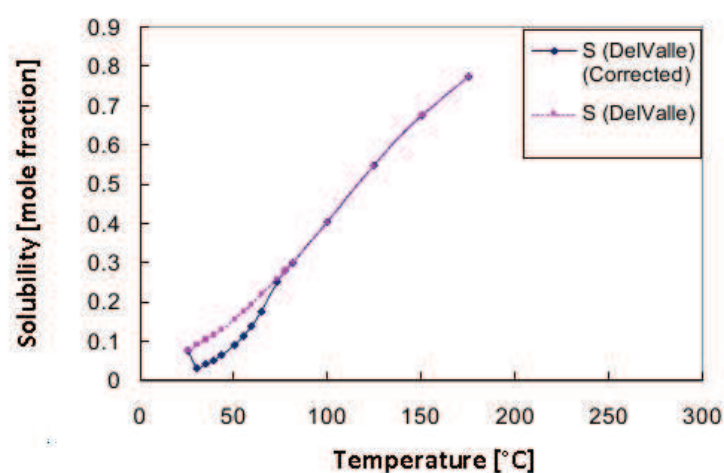


Figure 12.4 Erythritol solubility vs temperature using the equation of del Valle et al

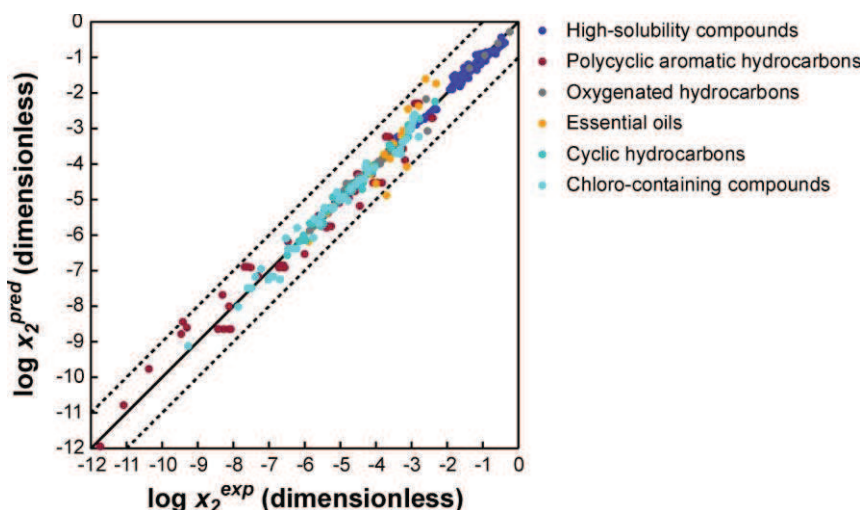


Figure 12.5 Predicted vs. experimental aqueous of del Valle et al.

12.4 Solubility parameter of naturally occurring polymers under sub-critical water conditions

Cellulose, hemicellulose and lignin represent the major constituents of lignocellulosic biomass, which as discussed in Chapter 2 is a dominant source of biomass. Therefore building on the empirical studies reported in Chapter 2 the solubility parameters were calculated for model polymers representing cellulose, hemicellulose and lignin with varying degrees of polymerisation. Figures 12.6 and 12.7 show the trend for cellulose over two distinct ranges of oligomerisation and temperatures from 25-300°C. As can be seen in Figure 12.6, most of the variation in polymer's solubility parameter occurs with oligomers with DP1-5 and the value asymptotically approaching a constant value with increasing chain length. Loss of the polymer's cohesion energy density is apparent with temperature; varying over 2.5 Hildebrand units for $n = 10$. Figure 12.7 verifies this trend over a much larger range of polymerisation, $n = 15,000$, which approximates the molecular weight ranges quoted for cellulose in biomass.

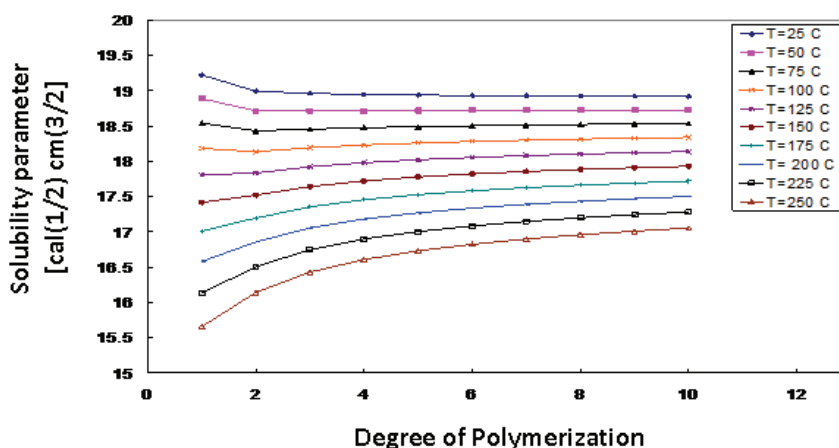


Figure 12.6 Variation of cellulose solubility parameter with DP/temperature

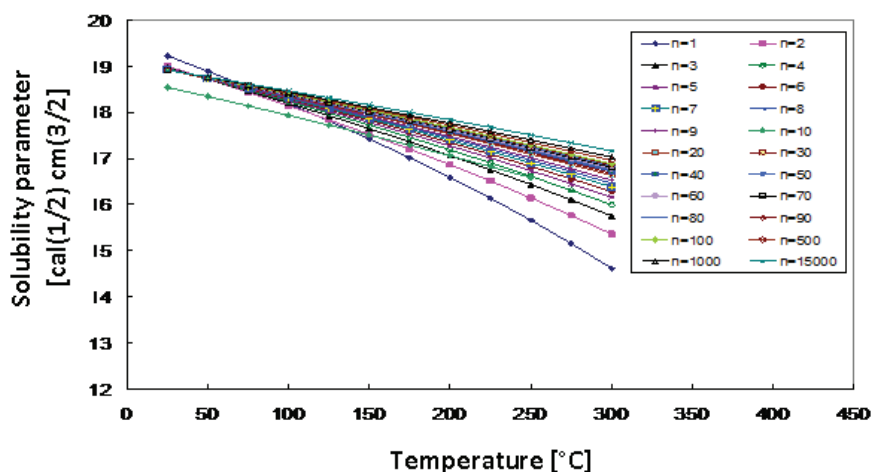


Figure 12.7 Cellulose solubility parameter as a function of temperature for longer DP

In a similar fashion, solubility parameters were calculated for lignin, hemicellulose, and a model polyflavonoid polymer (poly-catechin), and these are contrasted with those for cellulose in Figure 12.8 at 125°C. As illustrated in Figure 12.8 the model biopolymers exhibit different solubility parameters trends as their DP increases. For poly-catechin (calculated assuming the polymerisation occurs between the 4-8 linkages for consecutive catechin molecules), the cohesion energy density appears to increase before reaching a constant value at a chain length of $n = 10$ and beyond. For hemicellulose the model predicts that the polymer will be more soluble than cellulose over the same DP, indeed at the range of DP2-4 the xylose oligo saccharides (XOS) are noticeably more soluble a feature commonly reported from those working with XOS production and therefore supporting the model. The solubility parameter trend for cellulose and lignin is more monotonic, although these trends can change with temperature as shown in Figure 12.8 of particular interest are the relative values of solubility parameters for the four biopolymers. At 125°C, the polyflavonoid is more ‘polar’ on a relative basis than the three carbohydrate-based polymers. Interestingly, the cohesion energy density of lignin is considerably less than for the other three polymers. This suggests that lignin is not inherently more polar and hence difficult to dissolve with water from purely a solubility perspective; it’s just more recalcitrant with respect to its removal from biomass matrix. Hence when using sub-critical water as a processing solvent, the results in Figure 12.9 suggest that hotter water (less dense) would be preferred for this biopolymer versus less heated water for the other biomass constituents.

The objective of evaluating and developing solubility models was to enable the prediction of broadly optimal operating parameters to efficiently extract naturally occurring polymers from

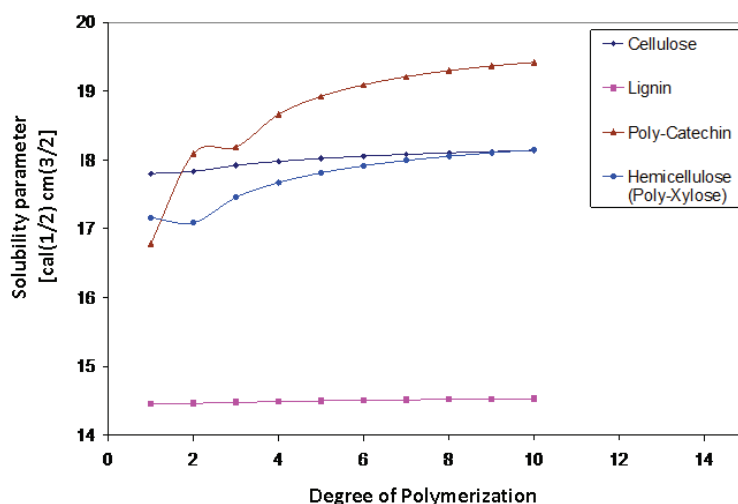


Figure 12.8 Variation in solubility parameter of biopolymer oligomers with DP at 125 °C

any source of biomass. The above data permits an estimation of the conditions for extracting (reacting) polymers such as lignin, hemicellulose and cellulose and visualise the impact of water's solubility parameter at various temperatures and reduced pressures on the solubility parameters of the target biopolymers.

This is conveniently done by overlaying the observed trends as a function of temperature for the biopolymers on the same graph (Figures 12.9 and 12.10). Again the solubility polymers for oligomers at $n = 1-10$ are used since these are the anticipated species formed in the water phase from exhaustive hydrothermal degradation of the biopolymer. The water solubility parameter-temperature plots are for $z_c = 0.23$ at $p_r = 0.01, 0.5$, and 1.0 (the critical pressure). As shown below for cellulose-water system in Figure 12.9, the water-solubility parameter line intersects the individual lines for the solubility parameters of the polymers. At this point, the degree of solubilisation of the biopolymer can be affected, or within 2.5 Hildebrand units of the solubility parameter defined by the intersection point. The point at which water's solubility parameter drops, as indicated in both Figures 12.9 and 12.10 reflects its transition to the vapour state (steam) due to inadequate pressure being applied to keep it in its hot liquid state. In terms of the loss of cohesion energy, subcritical water at $p_r = 0.01$ drops from a value of $20.75 \text{ cal}^{1/2}/\text{cc}^{3/2}$ at 115°C to $0.04 \text{ cal}^{1/2}/\text{cc}^{3/2}$ at 180°C , intercepting the cellulose solubility parameter loci at approximately 150°C and $14.35 \text{ cal}^{1/2}/\text{cc}^{3/2}$. However using sub-critical water under these conditions for an extraction-reaction is difficult due to the abrupt loss of cohesion energy density with temperature. For the case of cellulose, $p_r = 1.0$ (about 22 MPa), processing of cellulose with sub-critical water can be affected better between $180-295^\circ\text{C}$ since its cohesion energy density is equivalent to that for the cellulose oligomers over the extended temperature range. For the lignin model polymer (Figure

12.10), the situation is quite different due to the high cohesion energy density for water over an extended temperature range and the somewhat lower solubility parameter for lignin over the same temperature range. Extraction-reaction in this case is best carried between 322-335°C using a $p_r = 1.0$, however solubilisation can also be achieved between 250-300°C at a $p_r = 0.5$ (110 bar) using the 2.5 Hildebrand rule as a miscibility criterion. Preferred conditions also exist for hemicellulose as noted in Figures 12.11 where temperature ranges of 200-350°C at a $p_r = 1.0$ will permit solubilisation. At a $p_r = 0.5$, a more limited temperature range is applicable with respect to solubilising the lower oligomers.

We have used the solubility model (equation 12-4) on the hydrolysis of rice bran as discussed in Chapter 9 and in Figure 12.9 and 12.11 works done by Muhammad et al. shows the solubility temperature of cellulose and hemicellulose content of rice bran which shows a good correlation.

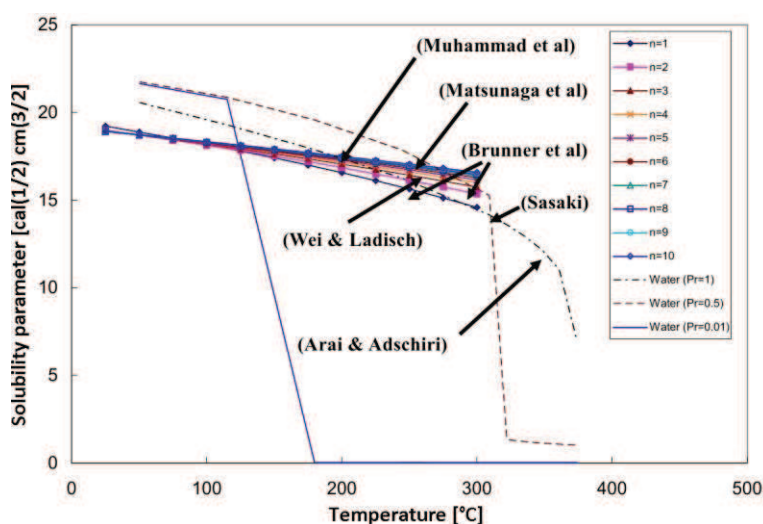


Figure 12.9 Solubility parameter variation for sub-critical water at different p_r and cellulose oligomers as a function of temperature

The above approach can also be used to predict the best extraction temperature for partitioning carbochemicals into hot compressed water. As shown below in Figures 12.12 and 12.13, extraction and fractionation of these compounds is possible at two different reduced pressures for water, a p_r of 0.2 and 0.01, respectively. From an applied perspective, extraction or fractionation at a $p_r = 0.01$ is more desirable since the required temperature is lower which is an advantage in extracting thermally-labile compounds.

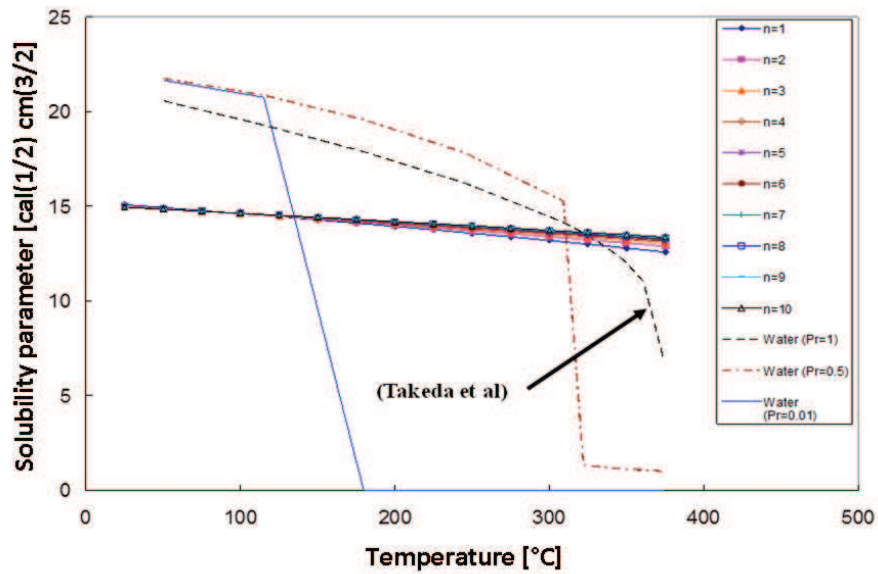


Figure 12.10 Solubility parameter variation for sub-critical water at different p_r and lignin oligomers as a function of temperature.

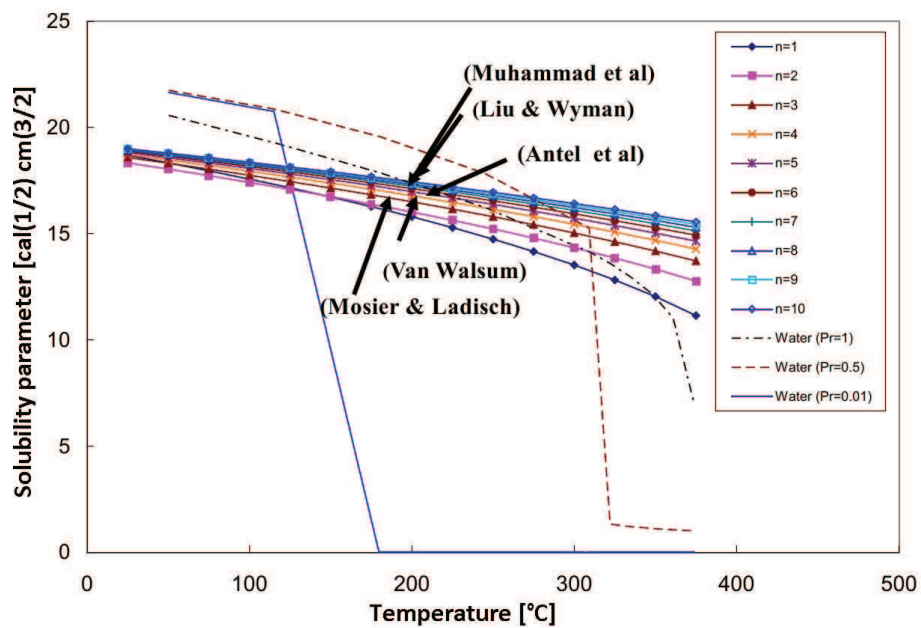


Figure 12.11 Solubility parameter variation for sub-critical water at different p_r and hemicellulose as a function of temperature

In addition there is a 25°C fractionation range as opposed to 15°C interval for the case when water is at a $p_r = 0.2$. The predicted temperature range of 125-150°C for water at a $p_r = 0.01$ is consistent with conditions for the extraction and recovery of anthocyanins devoid of thermal degradation (King et al., 2003; Yu and Howard, 2005) and natural products with sub-critical water (King et al., 2005b). We have also applied this approach at triacylglycerides as shown in Figure 12.14 which shows the complete solubility at temperature of 370°C.

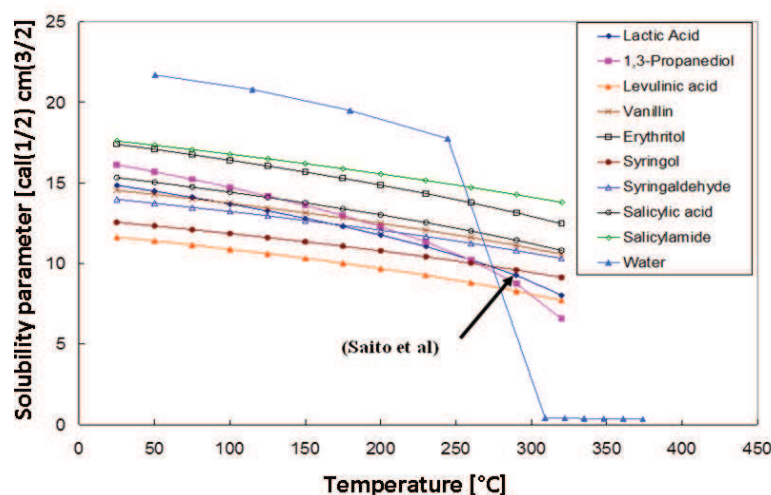


Figure 12.12 Prediction of the temperature range for extracting carbochemicals at a $p_r = 0.2$ with sub-critical water

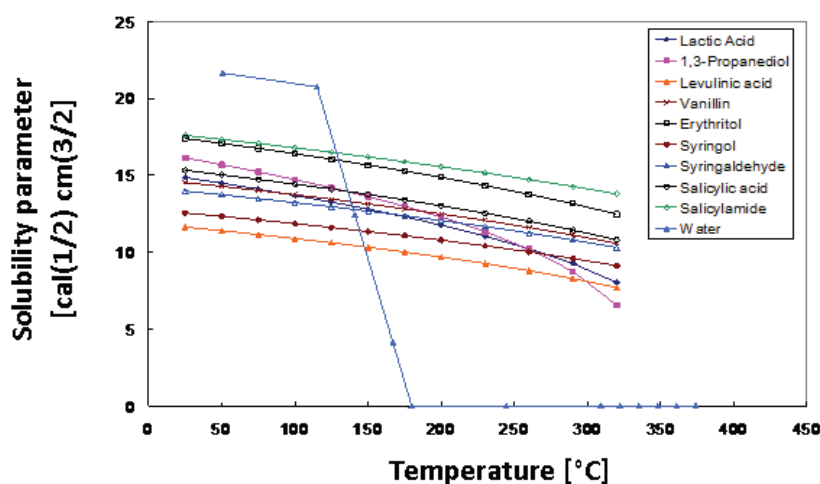


Figure 12.13 Prediction of the optimal extraction conditions for carbochemicals recovery using sub-critical water at a $Pr = 0.01$

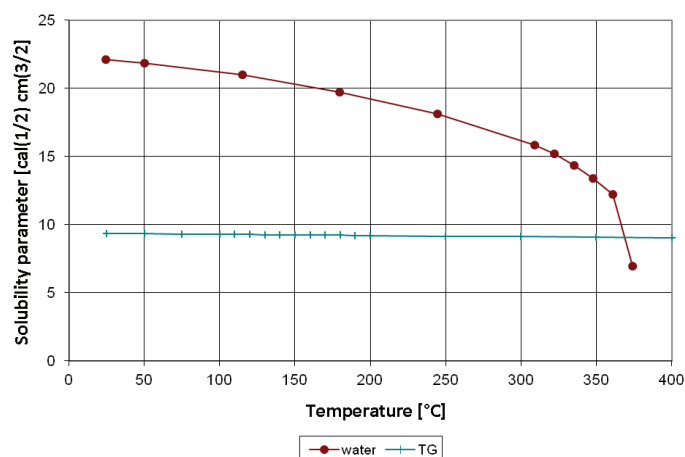


Figure 12.14 Prediction of the optimal solubility conditions for TAG using sub-critical water at a $Pr = 0.01$

12.5 Conclusion

In this study, a correlative method has been developed for predicting the solubility of organic solutes as a function of temperature in high temperature water. The correlative methods has been shown to be applicable to a diverse number of solute types up to temperatures of 400°C. It can be used to correlate the temperature dependence of solute solubility where fundamental physicochemical property data is not available. We found a good correlation of this solubility model with the work presented on rice bran and sunflower oil in previous chapters.

Methods for estimating the changes with temperature on solute solubility in water are needed for applications of hot, pressurized water as an extraction solvents or reaction medium. There is little data available and few correlative methods for estimating solute solubility under these conditions therefore the limited existing methods are based on limited solubility data base, and in some cases predicted solubility values are in quite serious disagreement with experimentally-derived data. Here available solute solubility data both above and below the boiling point of water has been correlated for diverse solute types consisting of hydrocarbons, essential oil components, pesticides, flavanoid-type compounds, as well as solutes exhibiting high solubility in water under the stated conditions. Conditions for unit operations commonly employed in biomass transformation and bio-refineries are frequently conducted under sub-critical conditions where $Tr = 0.50$ - 1.00 and $Pr = 0.02$ - 2.0 . This includes reactions conducted in sub-critical water and methanol, depolymerisation of natural biopolymers in aqueous media, and carbo chemical production in a ‘fermentation’ or ‘thermochemical’ based biorefinery. In this chapter we

present studies designed to optimise reactions and extractions conducted in the above media as a first step for integrating critical fluids into these biotransformation processes. Assessment of solute (reactant and/or product) solubility in the critical fluid medium is important in assuring phase miscibility, its influence on reaction rate and chemistry, and of course, extraction of the target solutes. Four equations have been tested with respect to their ability to predict solute solubility particularly in sub-critical water for a diverse group of solutes varying in chemical structure. Solute mole fractions in sub-critical water are found to vary from 10^{-9} – 0.5×10^{-1} , showing a pronounced increase with temperature above the boiling point of water. The magnitude of the expected change in solute solubility for over 40 solutes correlates approximately with their solubility parameter differences or their octanol-water coefficients, $K_{O/W}$. Temperature dependence of solute solubility can be modelled using the van't Hoff relationship advocated by Curren and King, (2001) for various classes of solutes. Utilization of this data will be described as applied to reactions and extractions conducted in sub-critical water. For example, saccharification reactions in sub-critical water conducted in the range of 100-130°C; the solubility for mono- and disaccharides are between 0.05–0.45 mole, being lower for $n=2$ oligomers, in this temperature region. Finally, it will demonstrate that extraction of value-added natural products, isolation of chemicals in bio-refinery production schemes and environmental remediation can be optimised using the reported data and approach for a diverse number of natural and commodity chemicals, leading to integrated processing schemes employing critical fluid media.

GENERAL CONCLUSION

The complexity of biomass chemical composition and its solid form impede the rational and complete use as a carbon source for chemical uses, whereas there is a growing need and demand of renewable feedstocks from consumers and industry.

Wood pulping for the paper industry, established more than a century ago, is the sole large scale process conducted on a continuous form; however the processes are severe generating significant wastes and evoking an environmental load. Currently industry is looking to develop and apply biotechnology to reduce the energy input, waste streams and therefore impact on the environment and climate. Today the prime example might be seen as bioethanol production i.e. fermentable sugars to yield one product, like ethanol for fuel or for ethylene, or the production of monomers like lactic acid for polymer. Therefore the company supporting this research work, that has developed its own integrated biorefining concept, continues to explore the utility of critical fluids. In particular the company is interested in developing technologies that enable the full utilisation of lignocellulosic biomass preserving the natural structures as much as possible while allowing the sequential extraction of all valuable compounds.

In the context of the company's ambitions the work addresses the first bottleneck i.e. the pre-treatment of biomass in a continuous flow mode, while adopting environmentally benign solvents. Critical fluids like CO₂, a readily available and overproduced gas, but also simply water, have been recognised as environmentally safe. Water is the most benign alternative (cheap, non-toxic, non-combustible) to organic solvents, and also a constituent of biomass. Supercritical water displays very interesting solvent and reactant properties, for example it is possible to vary its dielectric constant according to pressure and temperature, and the ion product constant is about three orders of magnitude larger (10^{-11}) than ambient water. However to achieve critical water requires elevated pressures and temperatures (374 °C and 22 MPa). In addition to support the use of supercritical water requires significant investment in equipment.

Fortunately some if not all of the properties exhibited by supercritical water are also accessible at lower temperature and pressure i.e. under subcritical conditions. Therefore the investigation of subcritical water using rice bran as a model substrate has shown its efficacy as medium to support biomass hydrolysis. The study has shown an almost complete hydrolysis of the hemicellulose component of rice bran, xylose being the main product after a residence time of 30-40 minutes at 200°C and 20 MPa. It is also possible to play with the complex reaction kinetics to obtain secondary reaction products like furanic derivatives (HMF, furfural) known and now used as “green” solvents, or even formic acid a commodity chemical. These interesting results obtained under catalyst-free continuous flow conditions are the result of the above mentioned special properties of water close to critical conditions.

The presence of acidic products like formic acid derived during the hydrolysis reaction will, it is thought as widely reported, contribute to the overall catalytic properties of water. The addition of CO₂ to subcritical water results in the formation of carbonic acid, a weak acid, and was shown to support improved rates of hydrolysis, especially at lower temperature. The resulting overall hydrolytic activity of subcritical water modified with CO₂, suggests that it is possible conduct reactions at lower operating temperature of about for example 180°C instead of 210°C. The computed overall activation energy shows the positive effect of adding CO₂.

Therefore this part of the work shows the possibility of selectively liquefying rice bran in a continuous processing mode, which was the target. In addition preliminary insights were gained as to the complex interplay between extraction (solubility), and catalytic properties of subcritical water but also the potential to modulate the overall efficacy of hydrolytic reactions towards hemicellulose.

The hydrolysis of vegetable oil to free fatty acid and glycerol form a central component of the well established oleochemical industry which exploits oleaginous biomass. Currently hydrolysis of lipids or ‘fat-splitting’ as it is referred is performed using water at 250°C and 5 MPa but more generally using steam. In this work we investigated the impact of elevate temperatures and pressures (270-350°C, 20 MPa) i.e. conditions closer to the critical point of water. The results showed, in comparison to current industrial fat splitting, complete hydrolysis of TAG could be achieved within 7-15 minutes, instead of hours. Therefore it was demonstrated that without addition of either a catalyst or CO₂ a FFA yield of 90% could be obtained. In addition, these conditions appeared to support further reactions of the primary

hydrolysis product, based on unknown peaks observed with increasing temperature, and even a drop of free fatty acid yield due to significant consumption by these reactions, although these products were not characterised, given the presence of C18:2 as main FA, it may be inferred from the literature that these could be FA dimers and polymers, already marketed for glue and paint applications. Therefore from an applied stand point, these conditions may provide a tunable process towards either commodities like FFA and glycerol, or higher value specialty oleochemicals.

On the chemical side, it has been found that the yield of primary hydrolysis products (FFA) is dependent on temperature, reaction time, water:oil ratio, and water density. A high yield and selectivity for FFA requires a longer residence time, but at moderate temperature (300°C or less) to avoid forming FA derivatives. The non targeted catalytic effects, mentioned in the literature, was measured through the pH of the crude hydrolysis product out of the reactor: high TAG conversion and low pH are strongly linked (however the involved acidic species were not identified). In addition, the peculiar properties of water may speed up the kinetics due to high ionic product, as catalyst, but also for stabilising the charged reaction intermediates. Finally, the oil hydrolysis reactions exhibited an induction period: during the early phases of oil hydrolysis the reaction medium is changing by inference from work which we have not shown using rice bran oil and tallow, fats with a high initial FFA content, it is possible to conclude that initial and liberated FFA add to the overall catalyst potential of the medium a phenomenon referred to as autocatalysis. Thus all this plays in favour of this process being versatile, accepting crude or high acidity oils, thus cheaper than refined oils.

These results provide valuable information for the optimisation under most favourable technical and economical conditions of this hydrolysis process based only on subcritical water in a continuous flow reactor, tuneable for one-step production of multiple products.

The last example of biorefining step is devoted to functionalisation of a primary product, i.e. the lipase catalysed synthesis of fatty acid ethyl ester. Reaction variables have been investigated and optimum conditions were determined under SCCO₂. The fact that the effect of pressure on the enzyme stability is quite small is a positive point, as well as the continuous configuration of reactor found to allow a faster reaction (~95% conversion in 60 min residence time, 20 MPa, ethanol concentration 2 M), compared to batch process (55% in 6 h with CO₂). Under batch mode using the same conditions, but without CO₂, the maximum conversion was only 38%, this equilibrated reaction being even more limited by the newly

formed water as a co-product. Here again this shows the positive effect of the CO₂. A more detailed knowledge of the reaction medium could help to shift the reaction to 100%.

As noted in the result chapters the solubility of natural compounds, either feedstocks or products, in the reaction medium is an important component when developing and designing processes, especially in subcritical water. The correlative method developed for predicting the solubility of organic solutes as a function of temperature in high temperature water, has been shown to be applicable to a diverse number of solute types up to 400°C. It can now be used to correlate the temperature dependence of solute solubility where when fundamental physicochemical property data are missing. A good correlation of this solubility model with the work presented on rice bran and sunflower oil was found.

In this work, an effort was made for modelling the behaviour of these complex systems, and the predicted models fit well with the experimental results, in spite of some noise resulting from introduced approximations.

During this work emphasis was placed on constructing complex pressurised units able to work under continuous flow, and on setting working conditions. Further chemical analysis of reaction products, and a deeper consideration of all possible chemical reactions and mechanisms, would now be necessary to obtain useful information for an improved efficiency of the reaction steps and yields.

As an example the water:oil ratio was seen to have a reverse effect depending on the setting of pressure in the course of the hydrolysis reaction of TAG. The continuous positive effect of pressure is more marked at low water:oil ratio, and appears to be dependent on the degree of hydrolysis. This observation would benefit from further investigations directed towards understanding the complex interactions between the many components in the reaction medium, water, TAG and the various products. For example it is to be expected that FFA and di- and monoacylglycerols play a role as known surfactants and therefore influence the colloidal structure of this complex multiphased or dispersed (micellar) reaction medium. Also the role of FFA as an acid catalyst, mentioned in the literature would warrant further study; similarly the observed pH lowering effect vs TAG conversion, would require further investigation, to identify the involved acidic species.

All this would then contribute to setting an integrated industrial unit able to play on the many advantages of the sub- and supercritical fluids, for biorefining biomass into multiple products.

Sub- and supercritical fluids offer environmental advantages over chemical solvents, whilst providing enhanced separation and chemical selectivity. Their use for the recovery of products from biomass and the transformation of selected molecules (to add value and functionality) was studied. The results support the use of critical fluids for process integration, as versatile environmentally-benign solvents and reaction media, and the objective of our sponsor to evaluate continuous flow sub-critical water and SCCO₂ for processing of natural feedstocks (here limited to a few biomass components), was achieved.

Continuous flow processes support reduced plant footprint, therefore offer advantages when considering large-scale production, whilst expected to maintain product quality owing to short residence time.

Finally, the by way of addressing the often posed question “What does it cost?” “it is cheaper” a primary economic analysis was conducted with the industrial partner, details are to be found in Appendix C. The results show that to produce FAEE from refined vegetable oil costs approximately €0.45/L. Although currently biodiesel is dominated by methyl esters (FAME) there has been considerable interest in evaluating FAEE as an alternative especially as using bioethanol would avoid the use of fossil fuel derived methanol. In the 2007 EU project BIOscopes (Hamelinck et al., 2007) was conducted to assess the utility of FAEE, and the assessment was positive with no inherent disadvantage, indeed at the time the FAME were estimated at €0.61/L while FAEE were estimated at €0.66/L. Obviously the use of less refined vegetable oils has increased lowering the unit price of biodiesel. However oils with a high initial FFA percentage require acid based chemistry and this in turn influences the downstream refining of glycerol. Therefore to conclude, in this work we have illustrated the utility of critical fluids to support economic production of FAEE which are emerging as a potential replacement for FAME in the biodiesel market, this point ignores the fact that FAEE currently have high value application in the cosmetic sector.

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Appendix A

Solvent	Reference	Lipase source											Support									
		Alcaligenes Sp.	Aspergillus sp.	Candida rugosa	Penicillium cepacia	Porcinepancreatic lipase	Pseudomonas cepacoi	Pseudomonas sp.	Rhizopus arrhizus	Rhizopus delemar	Rhizopus japonicus	Rhizomucor miehei	Celite	Filtercel	Hyflo Supercel	Glass Beads	Cellulose	PMMA (Novozyme)	Polystyrene	Polyethylene(Accurel) EP400	Polypropylene (Rigidex) HD	Polypropylene (Accurel) EP
Organic	Brady et. al. [1986]			■									■							■		■
	Geluk et. al. [1992]			■													■	■	■			
	Gray et. al. [1990]			■								■								■	■	■
	Goderis et. al. [1987]			■					■				■							■	■	■
	Montero et. al. [1993]			■																		■
	Wisdom et. al. [1984]		■										■	■	■							
Supercritical Fluid	Aaltonen & Rantakyla		■	■									■	■	■							
	Bornscheuer et.al. [1996]				■																	
	Chi et. al. [1988]	■								■	■		■									
	Erickson et. al. [1990]								■						■							
	Frykman et. al. [1998]						■					■			■							■
	Glowacz et. al. [1996]					■						■										
	Habulin et. al. [1996a]											■										
	Ikushima et. al. [1993; 1995b]			■								■				■						
	Kamat et. al. [1992; 1995a]			■												■						
	Kao et. al. [1997]			■																		
	Knez and Habulin [1992a]											■										
	Liu et. al. [1997]		■	■			■															
	Martins et. al [1994a]					■							■			■						
	Michor et. al. [1996]			■				■								■						
	Miller et. al. [1991]								■							■						
	Nakamura et. al. [1986]									■												
	Randolph et. al. [1988a]							■														
	Shishikura et. al. [1994]			■						■		■										
	Steytler et. al. [1991]			■												■						

Appendix A-1 Example of lipase and supports used for catalysis in both organic solvents and supercritical fluids (commercial immobilised biocatalysts are not included)

Supplier	Name	Lipase Source	Support	References
Novo Industri (Bagsvaerd, Denmark)	Lipozyme IM (type not specified)	Rhizomucor miehei	Duolite (Anionic resin)	Adschiri et. al. [1992], Berg et al. [1993] Bernard and Barth [1995], Castillo et al. [1994] Condoret et al. [1997], Dumont et al. [1992; 1993] Habulin et al. [1996a; 1996b; 1997] Marty et al. [1990; 1992; 1994; 1997] Shishikura et. al. [1994], van Eijs et. al. [1988]
	Lipozyme IM20	Native Rhizomucor miehei	Duolite (Anionic resin)	Berger and Schneider [1991], Brown et al. [1994] Chulalaksananukul et al. [1993] Chi et al. [1988], Claon and Akoh [1993] (hexane) Knez et al. [1990; 1995] Knez and Habulin [1992; 1994] Liu et al. [1997], Martins et. al. [1994a] Rantakyla et al. [1994; 1996] Rizzi et al. [1992], Vermue et al. [1992] Yoon et. al. [1996]
	Lipozyme IM60	Lipase from Rhizomucor miehei cloned into Aspergillus oryzae	Duolite (Anionic resin)	Claon and Akoh [1993] (hexane) Michor et al. [1996]
	Novozyme SP382	Native Candida antarctica	PMMA (acrylic resin)	Claon and Akoh [1993] (hexane)
	Novozyme SP435	Candida antarctica cloned into Aspergillus oryzae	PMMA (acrylic resin)	Claon and Akoh [1993] (hexane) Gunnlaugsdottir et al. [1997] Gunnlaugsdottir and Sivik [1995; 1997] Jackson and King [1996; 1997] Jackson et al. [1997] Michor et al. [1996]

Appendix A-2 Commercially available immobilised lipase biocatalysts

Reference	Solubility Data
Chrastil 1982	Stearic acid, oleic acid, behenic acid, tributyrin, triolein, palmityl behenate, behenyl behenate, α -tocopherol, cholesterol, water and cafestol (and correlation using a derived equation)
Bamberger et al., 1998	Lauric acid, myristic acid, palmitic acid, trilaurin, trimyristin, tripalmitin and mixtures of the three triglycerides (and correlation using a lattice model equation of state).
Iwai et al., 1996	Fatty acid and long chain alcohols (and correlation of using the virial equation of state)
Liong et al., 1992	Ethyl esters of the fatty acids C18:1, C20:3, C20:4 and C22:6
Martins et al., 1994	Glycidol at various concentrations of butyric acid
Marty et al., 1992	Oleic acid at various concentrations of ethanol
Nilsson and Hudson, 1993	Oleic acid, Palmitic acid and their triglycerides
Yu et al., 1992a	Oleic acid at various concentrations of ethanol
Yu et al., 1992b	Oleic acid, methyl oleate and anhydrous milk fat
Yu et al., 1992c	Anhydrous milk fat, rapeseed oil and soybean oil

Appendix A-3 *Relevant published solubility data for compounds in SCCO₂*

Reaction Type	References
Esterification	Bernard and Barth [1995], Castillo et al. [1994] Caussette et al. [1997], Dumont et al. [1992; 1993] Habulin et al. [1996a], Ikushima et al. [1996a; 1996b] Knez and Habulin [1992; 1994], Knez et al. [1995] Marty et al. [1990; 1992; 1994], Steytler et al. [1991] Yu et al. [1992a]
Acidolysis	Erickson et al. [1990], Liu et al. [1997] Miller et al. [1991], Nakamura et al. [1986] Shishikura et al. [1994]
Alcoholysis	Chi et al. [1988], Chulalaksananukul et al. [1993] Gunnlaugsdottir and Sivik [1995; 1997] Gunnlaugsdottir et al. [1997], Jackson and King [1996] Jackson and King [1997], Kamat et al. [1992; 1995a] Pasta et al. [1989], van Eijs et al. [1988] Vermue et al. [1992]
Transesterification	Adschiri et al. [1992], Berg et al. [1993] Brown et al. [1994], Jackson et al. [1997] Yoon et al. [1996]
Chiral Resolution	Bornscheuer et al. [1996], Capewell et al. [1996] Glowacz et al. [1996], Ikushima et al. [1993; 1995b] Martins et al. [1994a], Michor et al. [1996] Rantakyle et al. [1994], Rantakyla et al. [1996]

Appendix A-4 Reactions investigated in SCCO_2

Appendix B

Mass Balance Calculations

☐

☐

Lipase Catalysed Esterification Reaction in SCCO₂

A reaction¹ using the continuous packed bed reactor (Figure B-1) will be studied under the conditions presented below:

☐

☐

Immobilized lipase, Lypozyme IM: 5mg

Pressure: 10 MPa

Temperature: 40°C

Initial oleic acid concentration: 7.89 mM

Initial ethanol concentration: 42 mM

It is therefore the aim to determine the initial rate of reaction (or activity) per unit mass of biocatalyst.

☐

$$-V_{OA} = \frac{Q_{SCCO_2} C_{OAi} X_{OA}}{m} \quad \text{Equation B-1}$$

☐

☐

At low substrate concentrations it is assumed that the overall volumetric flow rate throughout the reactor is that of the solvent. Data is therefore required that relates solvent flow rate, Q_{SCCO_2} , with conversion, X_{OA} , for a given mass of biocatalyst, m , and initial oleic acid concentration, C_{OAi} . A mass balance is used to determine solvent flow rate.

Mass Balance

Figure B-1 is a simplified process diagram of the high pressure continuous packed bed reactor.

¹Example based upon experiment No. 2, carried out on 20th Sep 2007

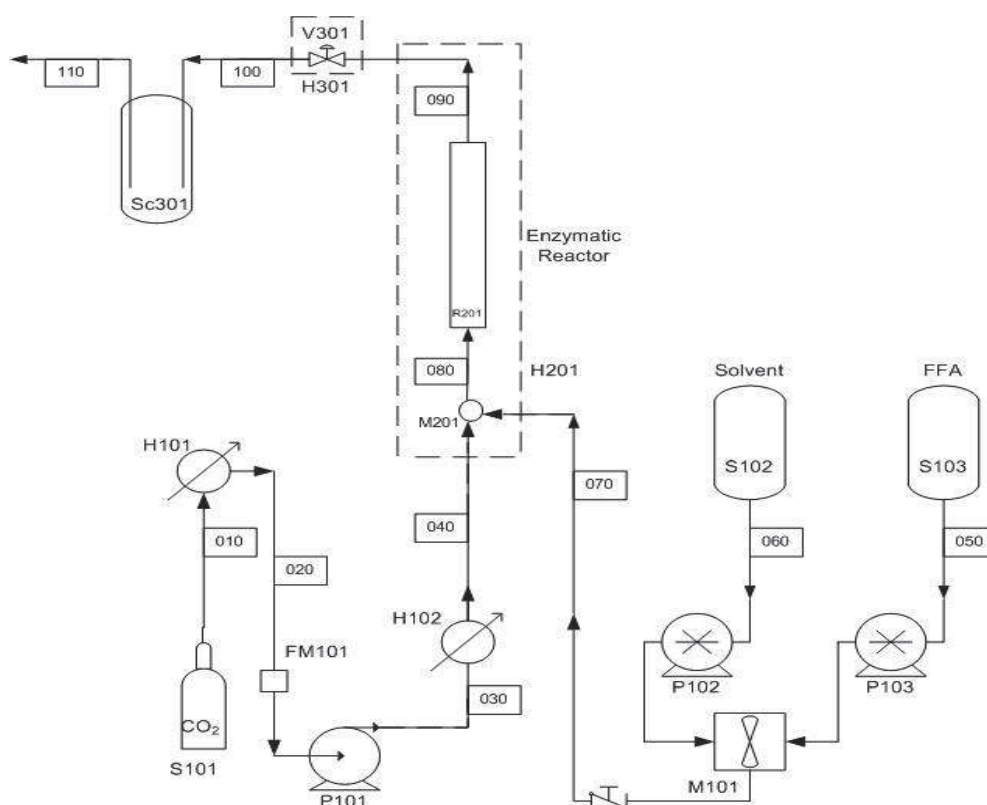


Figure B-1 S101, Liquid CO₂ cylinder; H101, Chiller; FM101, Flowmeter; P101, High pressure dual piston pump; H102, Thermostatically controlled heater; S102, Solvent storage; S103, Substrate storage; P102 & P103, Gilson 305 HPLC pump; M101, Mixture; R201, Packed Bed Reactor; V301, back pressure regulator; H301, Heated jacket; Sc301, Collection vessel.

A mass balance is required to predict the required substrate flow rate for a given concentration and solvent flow rate. Such a balance is also required to determine the solvent volumetric flow rate at system pressure and temperature. A rotameter located after stream 110 may be used to measure the volumetric flow rate of CO₂ gas exiting the reactor at atmospheric pressure and temperature and converted to system conditions using density data (Angus et al., 1973). For simplicity, a liquid CO₂ flow rate basis will be made in this example:

For a basis of 4.7 mL min⁻¹ liquid CO₂, determine:

- The required total substrate flow rate (oleic acid, ethanol -Stream 070).
- The SCCO₂ flow rate at system pressure and temperature (Stream 040).
- The CO₂ flow rate at atmospheric pressure and temperature exiting the reactor (Stream 110).

Stream 020

Description: Chilled liquid CO₂ at approx. -5°C and 5MPa (cylinder pressure).

Data: Liquid CO₂ density = 1.101 g/mL at 0°C, 0.1MPa (Perry et al., 1978)
(assumed incompressible).

Calculations: Mass flow rate = Volumetric flow rate x Density

Table B-1 Mass Balance for stream 020

Component	Temperature [°C]	Pressure [MPa]	State	Volumetric Flow rate [mLmin ⁻¹]	Mass flow rate [gmin ⁻¹]
CO ₂	-5	5	Liquid	4.7	4.7x1.101=5.2
TOTAL	-	-	-	4.7	5.2

Stream 030

Description: Compressed liquid CO₂ at approximately 5 to 10°C and 10 MPa

□

Stream 040

Description: Supercritical CO₂ at 40°C and 10 MPa

Data: SCCO₂ density = 0.776 g/mL at 40°C, 10MPa (Angus et al., 1973)

Calculations: Volumetric flow rate = Mass flow rate / Density

□

Table B-2 Mass Balance for stream 040

Component	Temperature [°C]	Pressure [MPa]	State	Volumetric Flow rate [mLmin ⁻¹]	Mass flow rate [gmin ⁻¹]
CO ₂	40	10	Fluid	5.2/0.776=6.7	5.2
TOTAL	-	-	-	6.7	5.2

Stream 050 & 060

Description: Substrate stream containing oleic acid, ethanol at atmospheric pressure and temperature (substrate flow rates based on concentrations specified above).

Assumptions: a) Substrates are all incompressible.
b) Overall flow rate based on SCCO₂ stream 040.
c) Negligible change in molar volume upon mixing.

Data: a) Oleic acid density = 0.891 g/mL at 20°C, 0.1 MPa [data from Sigma Chemical Co.]

b) Ethanol density = 0.789 g/mL at 20°C, 0.1 MPa (Perry et al., 1978)

Calculations: Molar flow rate = Concentration x Total volumetric flow rate
 Mass flow rate = Molar flow rate x Relative molecular mass

Table B-3 Mass Balance for stream 050 & 060

Component	Temp [°C]	P [MPa]	State	Conc. [mM]	Molar flow rate ($\mu\text{mole}/\text{min}^{-1}$)	Mass flow rate [gmin^{-1}]	Volumetric Flow rate [mLmin^{-1}]
Oleic Acid	20	0.1	Liq	7.89	$7.89 \times 6.7 = 52.86$	$(52.86/1 \times 10^6) \times 282 = 0.014$	$0.014/0.891 = 0.016$
Ethanol	20	0.1	Liq	42.0	$42 \times 6.7 = 281.4$	$(281.4/1 \times 10^6) \times 46 = 0.012$	$0.012/0.789 = 0.015$
TOTAL	-	-	-	-	615.66	0.026	0.031

Stream 070

Description: Substrate stream containing oleic acid, ethanol at system pressure and temperature (substrate flow rates based on concentrations specified above).

Assumptions: Substrate are all incompressible.

Stream 080

Description: Solution containing substrates and SCCO_2 at operating pressure and temperature.

Assumptions: Solution is perfectly mixed.

Table B-4 Mass Balance for stream 080

Component	Temp [°C]	P [MPa]	State	Conc. [mM]	Mass flow rate [gmin^{-1}]	Volumetric Flow rate [mLmin^{-1}]
Oleic Acid	40	10	Solution	7.89	0.014	0.016
Ethanol	40	10	Solution	42.0	0.012	0.015
CO_2	40	10	Solution	-	5.20	6.70
TOTAL	-	-	-	-	5.22	6.73

Stream 090

Description: Solution exiting reactor containing substrates, products (from reactor) and SCCO_2 at operating pressure and temperature.

□

Assumptions:

- Negligible pressure drop occurs across the bed (reasonable as only 5 mg of biocatalyst is used).
- The system is at steady state, *i.e.* all products (ethyl oleate and water) produced from the reaction exit in stream 090.

- c) An oleic acid conversion of 15% has been achieved with 5mg of biocatalyst. (from titration calculation).
- d) Volumetric flow rate remains constant through the reactor.

Data: Ethyl Oleate density = 0.800g/mL at 20°C, 0.1MPa (data from Sigma Chemical Co).

□

Calculations: Product conc. = Oleic acid conversion x initial oleic acid conc.

Table B-5 Mass Balance for stream 090

Component	Temp [°C]	P [MPa]	State	Conc. [mM]	Molar flow rate ($\mu\text{mole}/\text{min}^{-1}$)	Mass flow rate [gmin^{-1}]	Volumetric Flow rate [mLmin^{-1}]
Oleic Acid	40	10	Solution	$7.89-0.15 \times 7.89 = 6.70$	$6.70 \times 6.7 = 45$	$(45/1 \times 10^6) \times 282 = 0.012$	0.016
Ethanol	40	10	Solution	$42-1=41$	$41 \times 6.7 = 275$	$(275/1 \times 10^6) \times 46 = 0.012$	0.015
Water	40	10	Solution	1	$1 \times 6.7 = 6.7$	$(6.7/1 \times 10^6) \times 18 = 0.0001$	0.0001
Ethyl Oleate	40	10	Solution	1	$1 \times 6.7 = 6.7$	$(6.7/1 \times 10^6) \times 310 = 0.002$	$0.002/0.800 = 0.003$
CO ₂	40	10	Solution	-	-	5.2	6.7
TOTAL	-	-	-	-	-	5.22	6.73

Stream 100

Description: Liquid/gas mixture expansion valve at 60°C (Heated Jacket, H301) and atmospheric pressure.

Data: CO₂ gas density = 0.001755 g/mL at 60°C, 0.1MPa (Angus et al., 1973)

Table B-6 Mass Balance for stream 100

Component	Temp [°C]	P [MPa]	State	Volumetric Flow rate [mLmin^{-1}]	Mass flow rate [gmin^{-1}]
Oleic Acid	60	0.1	Liquid	0.016	0.012
Ethanol	60	0.1	Liquid	0.015	0.012
Water	60	0.1	Liquid	0.0001	0.0001
Ethyl oleate	60	0.1	Liquid	0.003	0.002
CO ₂	60	0.1	Gas	$5.2/0.001755=2963.0$	5.2
TOTAL				2963.03	5.22

Stream 110

Description: CO₂ gas exiting knockout pot, Sc301, at atmospheric pressure and temperature.

Data: CO₂ gas density = 0.001755 g/mL at 23°C, 0.1MPa (Angus et al., 1973)

Assumptions: a) All liquids have been removed from the gas stream.
b) All CO₂ gas exits the knockout pot, Sc301.

Table B-7 Mass Balance for stream 110

Component	Temp [°C]	P [MPa]	State	Volumetric Flow rate [mLmin ⁻¹]	Mass flow rate [gmin ⁻¹]
CO ₂	23	0.1	Gas	5.2/0.001755=2963.0	5.2
TOTAL				2963	5.2

A rotameter has been used to determine the CO₂ gas flow rate.

Oleic Acid Conversion

The conversion is calculated using the initial oleic acid concentration (equivalent or otherwise) and the concentration in the exit stream, 080 (equivalent or otherwise):

$$X_{OA} = \left(1 - \frac{6.70}{7.89}\right) \times 100 = 15\%$$

It is now possible to calculate the rate of reaction for the conditions specified

$$-V_{OA} = \frac{6.7 \times 7.89 \times 0.15}{0.005} = 1585 \mu\text{moles} / \text{min} / g_{cat}$$

This value represents the rate of production of ethyl oleate per unit mass of biocatalyst.

Appendix C

SIMULATION, ECONOMICS & COSTING

High pressure transesterification process for the production of fatty acid esters from vegetable oils has been described in general terms (Zhang et al., 2003) recently presented a process design and technological assessment of biodiesel production from both virgin vegetable oil and waste cooking oil at near ambient pressures.

The objective of this work is the evaluation of biomass derived components as a biorefinery concept coupling hydrolysis and enzymatic esterification, hence two step process to produce esters using a model substrate as a sunflower oil. Hence this work is based on modelling and optimization of this process to generate estimated cost of ester (biodiesel) production. We have used a limited number of units to give a flavour to readers for this technology.

The reports generated will provide the estimated cost of biodiesel production based on assumptions, made by the authors, regarding production volume, feedstock, and chemical technology. There could be great value, however, in having a flexible model that allows the user to make changes in these variables and examine the impact of such changes on product cost. Since all studies to date have shown relatively high costs for biodiesel production, a flexible model could aid in the comparison of alternate production routes for their abilities to achieve a very desirable reduction in production costs. It could also highlight the costliest operations in a proposed production scheme, allowing the focus of cost reduction efforts where they might have the greatest impact. Such a model could thus assist in determining the overall economic feasibility of a proposed operation, and guide choices regarding feedstock, chemical process, plant capacity and design. We have designed such a model, describe here its features, and demonstrate its usefulness in estimating capital and production costs for the synthesis of biodiesel from sunflower oil.

Components of the model biomass recovery facility

General features of the design

Analysis The approach involved the design of a model industrial operation for biomass recovery, the assembly of data for the purchase and assembly of its components, and the estimation of its operating expenses, resulting in an estimate of esters (biodiesel) productions. Information on biodiesel production was collected from various technical sources, including engineering firms that provide biodiesel processing expertise, equipment suppliers, and researchers and practitioners experienced with this topic. In the choice of construction materials, the most economical of available options was

chosen. Thus, for example, storage tanks were specified to be constructed of carbon steel, while stainless steels were specified in other applications as appropriate.

The economic model was developed by methods generally used to prepare conceptual cost estimates from flowsheets, as recommended by the Association for the Advancement of Cost Engineering (1990). In the design of this model, material and performance parameters of each piece of equipment involved in the process were specified. Equipment costs were based on Richardson Process Plant Construction Estimating Standards (2001), Chemcost Capital Cost and Profitability Analysis Software (1990), information from equipment suppliers, and historical equipment costs from Superpro files. These values were then used to calculate total installed costs through the use of Installation Factors (Hand, 1992), which convert the supply costs of equipment to total installed costs. The total calculated installed cost also includes the equipment installation costs and the cost of all required piping, electrical and other materials for the functioning unit. Table C-1 lists the values chosen for various expendables, utilities, labor and other expenses.

Table C-1 Operating cost and revenue values employed in this study

Item	Cost (US\$)
<i>Raw material, utilities</i>	
Sunflower oil	0.55/kg
Ethanol	0.30/kg
Carbon dioxide	0.01/kg
Electricity	0.1/kW-h
Steam (high pressure)	0.02/kg
<i>Additional operating costs</i>	
Plant operating labor	2 Persons/shift
Plant operators base rate	11.50/hr
Labor fringe benefits	40% of total labor costs
Operating supplies	20% of operating labor
Maintenance supplies	1% of capital costs, annually
General and administrative	0.50% of capital costs, annually
Taxes-property	0.1% of capital costs, annually
Insurance	0.5% of capital costs, annually

A depreciable life of 10 years was assumed. The escalation rate was set at 1% annually. Economic factors not accounted for were: Internal rate of return, economic life, corporate tax rate, salvage value, debt fraction, construction interest rate, and long term interest rate.

Working capital, environmental control equipment, marketing and distribution expenses, the cost of capital, and the existence of governmental credits or subsidies were excluded from these calculations. The total capital cost for the facility will be impacted by the cost of working capital, the interest during construction, and the cost of pollution control equipment. The resulting model is intended to be generic, and representative of contemporary industry practices. It is not meant to represent the actual

biodiesel design offered by any single technology provider; also as described earlier we used a limited number of process equipment.

The design was based on the use as feedstock of sunflower oil with negligible free fatty acid content. The facility contained three processing sections as shown in Figure C-1.

- (1) Hydrolysis unit where the vegetable oil was subjected to hydrolysis process to produce fatty acid and co-product glycerol
- (2) Glycerol recovery section.
- (3) Conversion unit of fatty acids to esters (biodiesel)

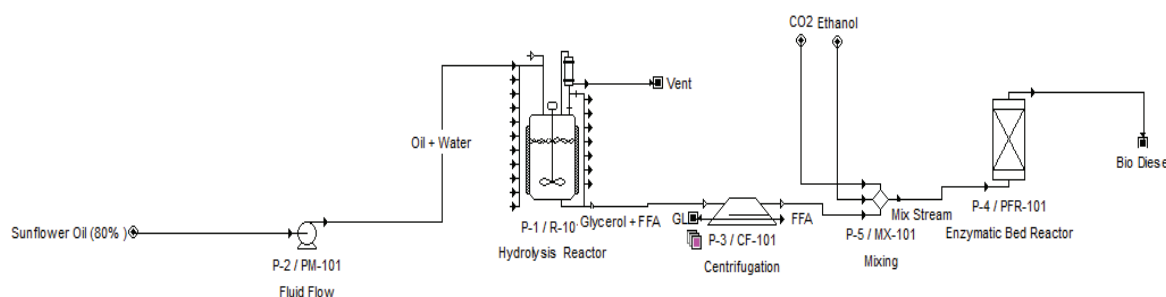


Figure C-1 Process flow diagram generated by super pro deign

Hydrolysis Reactor

Hydrolysis of sunflower oil used at a ratio of 80:20 was modeled as a continuous reaction conducted in a steam jacketed stirred tank reactor (Figure C-1, R-10) at 200°C for a residence time of 60 minutes. In a result of this reaction FFA's and glycerol are formed for extend of reaction of 99%.

Centrifugation

The mixture of FFA's and glycerol was fed to continuous centrifuge (Figure C-1, CF-101) which separated the glycerine and FFA's. For the production rate of 35,576,640 l of biodiesel, two centrifuge units required. The glycerol rich aqueous stream from this operation is sent to the glycerol recovery section while the impure FFA's is fed into a mixer (Figure C-1, MX-101)

Esterification -Enzymatic Bed Reactor

FFA's is mixed with CO₂ and ethanol in mixer (Figure C-1, MX-101) and then fed into Enzymatic bed rector (Figure C-1, PFR-101) where the FFA's is converted into esters in the presence of enzymes at working temperature of 60°C and pressure of 20 MPa for a residence time of 60 min. Water recovered during the process is recycled.

Analysis

Based on contemporary production processes and using current best values for reagent, equipment, and supply costs, a computer model of a esters (biodiesel) production facility was designed, and employed to estimate the capital and production costs for the synthesis of fuel grade biodiesel from

sunflower oil. This model is relatively preliminary in regard to the level of its detail. It is not meant to replace the thorough engineering analysis that is required in the final design and construction of such a plant, but rather is meant for use as a tool in estimating capital and operating costs. The model is flexible, and is meant for use in assessing the effects on estimated biodiesel production costs of changes in feedstock, in feedstock and glycerol prices, in chemical or process technology employed, or in equipment specified for the facility.

Based on the process flow diagram shown in Figure C-1, capital and production costs were calculated and are summarized in Table C-2. The estimated total capital cost was approximately US\$ 11.1 million. One third of this was for actual hardware, and two thirds was based on our assumption of a construction cost, roughly double the equipment costs.

The projected unit operating costs for the modeled biodiesel production facility are shown in Table C-3. This analysis calculates a final biodiesel production cost of US\$ 0.58/l. Raw materials costs constitute the greatest component of overall production costs, and of these the cost of the sunflower oil feedstock is the biggest contributing factor, itself constituting 90% of the overall production cost. These values are consistent with the results of other analyses of the costs of biodiesel production from refined sunflower oil (American Biofuels Association & Information Resources Inc., 1994; Bender, 1999; Graboski and McCormick, 1998).

Table C-2 Summary of capital cost and revenues

Investment	11,208,516 \$
Annual operating cost	36,541,781 \$/year
Main annual revenue	47,520,000 \$/year
Other annual revenue	2,213,137 \$/year
Total annual revenue	49,733,137 \$/year
Unit production cost	1.15 \$/kg
Unit production revenue	1.56 \$/kg
Gross margin	26.52%
ROI	78%
Payback time	1.28 year
IRR	54.61%
<i>Capital Investment</i>	
Equipment purchase cost	1,479,375 \$
Direct fixed capital	8,768,129 \$
Working capital	2,001,980 \$
Startup and validation cost	438,406 \$

The glycerol co-product generated during biodiesel production from a triacylglycerol feedstock was assigned a market value of US\$ 0.20/kg in this model, representative of its recent value when sold as a crude 80% aqueous solution. As biodiesel production volumes increase in the future it is expected that the concomitant increase in glycerol supplies will reduce its market value.

Table C-3 Unit costs for the annual production of 35,576,640 l of biodiesel from sunflower oil

Materials	0.634 \$/kg
Labor	0.012 \$/kg
Consumables	0.003 \$/kg
Utilities	0.014 \$/kg
<i>Total</i>	0.663 \$/kg

This model is meant as a research and planning tool. It is flexible in that elements of the scale, process or physical plant can be modified by the user to estimate the effects of changes in these parameters on capital and production costs. Also, it serves as the basis for future work, presently underway here, to estimate the cost of production of biodiesel from other feedstocks.

Conclusion

The spreadsheet model described here was developed to be used for research only. The authors do not accept responsibility for the accuracy of this program or decisions taken based on the model results. For specific applications of this spreadsheet, users should contact the authors for more detailed information, and information regarding the limitations and scope of the model.

The route we have chosen to convert the biomass into useful chemical products. For this purpose we have developed a computer model to estimate the capital and operating costs of a moderately-sized industrial biomass recovery plant. The major process operations in the plant were continuous-process vegetable oil hydrolysis and esterification, and fatty acid and glycerol recovery. The model was designed using contemporary process simulation software, and current reagent, equipment and supply costs, following current production practices. Sunflower oil was specified as the feedstock. Annual production capacity of the plant was set at 35,576,640 l. Facility construction costs were calculated to be US\$11.1 million. At a value of US\$ 0.55/kg for feedstock sunflower oil, a biodiesel production cost of US\$ 0.58/l was predicted. The single greatest contributor to this value was the cost of the oil feedstock, which accounted for 90% of total estimated production costs. Process economics included the recovery of coproduct glycerol generated during biodiesel production, and its sale into the commercial glycerol market as an 80% w/w aqueous solution, which reduced production costs by 6%. The model is flexible in that it can be modified to calculate the effects on capital and production costs of changes in feedstock cost, changes in the type of feedstock employed, changes in the value of the glycerol coproduct, and changes in process chemistry and technology.

Appendix D

Main Publications



Evaluation and modelling the utility of SCCO₂ to support efficient lipase mediated esterification

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ABSTRACT

Supercritical fluids offer environmental advantages over chemical solvents, while providing enhanced separation and chemical selectivity. The use of supercritical fluids for the recovery of products from biomass and the transformation of selected molecules (to add value) was studied. Free fatty acids were bio-catalytically transformed to fatty acid esters using lipase within a supercritical fluid environment. A central composite rotatable design was used to evaluate the influence of operating conditions on the enzymatic esterification process and a response surface equation was optimized to identify the most favourable process conditions for maximum free fatty acid conversion. Based on the model equation the process conditions under which it was predicted a yield of 100% esters could be obtained were: pressure 200 bar, temperature 60 °C, ethanol concentration 2.0 M, enzyme concentration 11 wt.% and time 60 min. Experiments conducted under these conditions gave an ester yield of 94.3% (close to predicted results). The activity per unit mass of biocatalyst was found to be 1585 μmol/min/g_{cat}. The results support the use of supercritical fluids for process integration.

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1. Introduction

A bio-based economy is the stated vision for the 21st century. Underpinning this goal is increased utilisation of biomass to create industrial feed stocks thereby loosening the current dependence on fossil fuel. Integrated biorefining is a broad all-encompassing term which describes the ambition of achieving full utilisation of primary agricultural biomass or organic waste and creating platform chemical, biofuels and materials [1]. To support this goal requires bio-processing technology development, which in turn evokes the selection of solvents. Supercritical fluids (SCF) are recognised as environmentally 'green solvents'. SCF are any substances at temperatures and pressures above their thermodynamic critical point. For carbon dioxide the critical point is 73.8 bar and temperature 31.1 °C. Supercritical carbon dioxide (SCCO₂) has emerged as an environmentally benign substitute for more conventional solvents and has been investigated for its suitability to support reaction medium [2], it has also been used extensively as a solvent for lipase catalyses reactions [3–5]. It has already been shown to provide a stable and effective environment for synthesis and separation.

However, the generally low solubility of compounds in SCCO₂ limits any kinetic advantage. If the solvent is completely removed then mass transfer limitations are likely to be more dominant and rates of reaction should improve due to higher substrate concentration [6].

Biocatalysts offer significant environmental and economic benefits over conventional chemical methods of manufacture, processing due to milder reaction conditions, increased reaction efficiencies, selectivity reduces the number of process steps, and decreases use and disposal of hazardous substances [7]. The use of biocatalysts in organic solvents offers many advantages over using aqueous media. Among these media SCCO₂ exhibits properties similar to organic solvents, but with the additional capacity of transport phenomena and facilitating reaction products separation by tuning the solvent power, thus the combination of SCCO₂ and enzymes offer significant potential with regards to developing environmentally responsible bio-processing.

Despite the existing work on lipase performance in SCCO₂ there are only a few studies which undertake the evaluation of the impact of multiple variables in a continuous flow mode relevant to process development [8]. To address this issue and at that same time limit the number of empirical experiments response surface methodology (RSM) which enables the evaluation of the effects of multiple parameters, alone or in combination, on response variables and also

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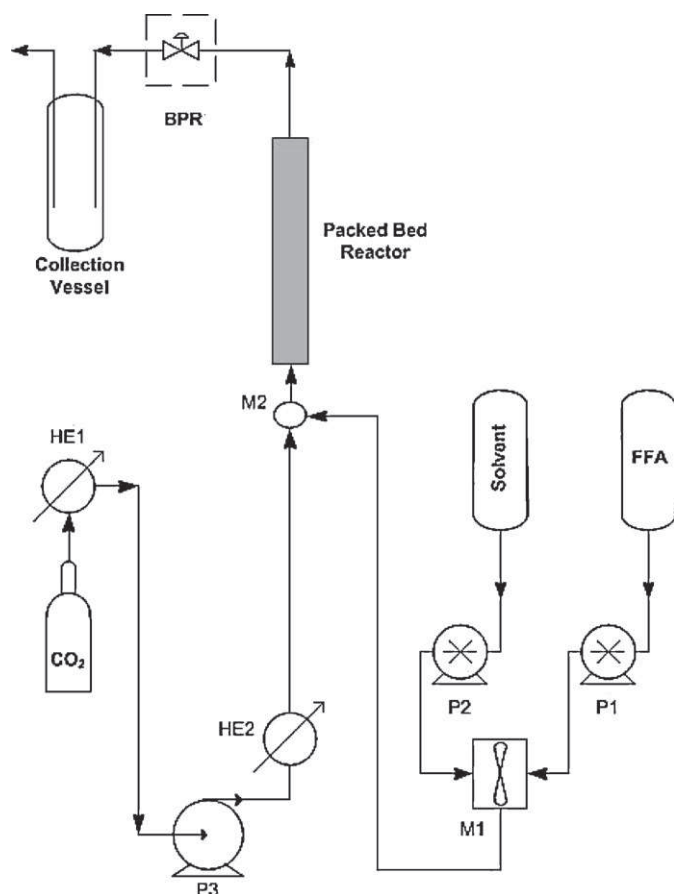


Fig. 1. Continuous flow esterification in packed bed enzymatic reactor.

predicts their behaviour under a given set of conditions have been adopted [9] and successfully validated.

In light of this, the objective of the research is to demonstrate the utility of supercritical fluids supporting the enzyme mediated transformation of fatty acids. To achieve this objective a five factorial response surface model was designed for the continuous flow lipase mediated ethanolysis and validated. The result will be presented using existing knowledge to address the impact of each parameter.

2. Materials

The esterification experiments were conducted with 89% pure oleic acid (Sigma Aldrich) and an immobilised lipase, Lipozyme TL IM® (Novozyme) from *Thermomyces lanuginosus* with specific activity of 250 IUN/g. Carbon dioxide (99.99%) was purchased from BOC (UK). The reagents used were ethyl alcohol (99.99%), sodium hydroxide (98.0%) and hexane (99.99%) from Fisher Scientific.

3. Methods

3.1. Enzymatic esterification of free fatty acid in the batch process

Three solutions with enzyme concentrations 5, 10 and 15 wt.% of 50 mM oleic acid were prepared. These were each combined with 100 mM of ethanol and 15 ml of hexane in a 50 ml conical flask and placed in a shaking water bath (GFL-Germany) at a rotation rate of 300 rpm and temperature of 55 °C. Samples of each were taken after 2, 4, 6 and 24 h. The solution with a 10 wt.% of enzyme was additionally studied for the reaction on addition of CO₂.

3.2. Enzymatic esterification of free fatty acid in a continuous flow process

Continuous enzymatic esterification was carried out in a packed bed reactor (TharSFC, USA) loaded with lipase, Lipozyme TL IM® (Novozyme) through which a mixture of oleic acid, ethanol and SCCO₂ was pumped (Fig. 1) at different process conditions (Table 1). Mixer – M1 used to ensure that the solvent and substrate are completely mixed. All reactions were carried out in a 100 ml reactor with a working

temperature of up to 200 °C, pressure of 300 bar and loaded with enzyme. CO₂ was pumped via P3 into the reactor. A sample collection vessel was used at the exit to the reactor. A backpressure regulator was used to maintain pressure in the system and an internal heating system used to reduce the likelihood of CO₂ sublimation.

3.3. Determination of free fatty acid and fatty acid ethyl esters content

The concentration of fatty acids ethyl esters were analysed by gas chromatography model HP 5890A equipped with flame ionisation detector, a HP 7673C auto sampler and a HP 3396B integrator using DB-5 capillary column (J&W Scientific, CA) with dimension 30 m × 0.25 mm and film thickness of 0.1 μm. Split ratio was 1:50, oven temperature 10 °C/min from 150 °C (2 min) to 280 °C (3 min). Injection temperature was 280 °C and detector temperature was 300 °C, injection volume was 1 μl and carrier gas was nitrogen.

The percentage conversion (%) of fatty acid was measured by determining the remaining un-reacted fatty acid in the reaction mixture by titration using the AOCs official method Ca 5a-40 [10]. The results were also verified by the photometric technique using MicroChem II analyzer by SafTest system (MP Biomedicals, UK). Free fatty acids (FFA) were determined spectrophotometrically with a chromogen reagent interacting with the FFA and changing its visible spectrum. This is a primary method calibrated with known amounts of FFA. The limit of detection (LOD) was 0.02%.

3.4. Rate of biosynthesis of ethyl oleate per unit mass of biocatalyst

The reaction was carried out using a continuous packed bed reactor (Fig. 1) with 5 mg of Lipozyme IM, at 100 bar, 40 °C, 7.89 mM oleic acid and ethanol concentration of 42 mM. The aim was to determine the initial rate of reaction (or activity) per unit mass of biocatalyst by using Eq. (1), which is a form of the steady state plug flow model [11]. It assumes that the density of the medium is constant at all conversions and that axial (forward and backward) mixing is negligible. Alain [12] has demonstrated that the esterification of oleic acid with ethanol in a continuous packed bed reactor may be satisfactory described by the Ping-Pong Bi-Bi mechanism [13] using a steady state plug flow model.

$$-V_{OA} = \frac{Q_{SCCO_2} C_{OAI} X_{OA}}{m} \quad (1)$$

At low substrate concentrations it was assumed that the overall volumetric flow rate throughout the reactor was that of the solvent. Data was therefore required that related solvent flow rate, Q_{SCCO_2} with conversion, X_{OA} for a given mass of biocatalyst, m and initial oleic acid concentration, C_{OAI} . Mass balance was used to predict the required substrate flow rate for a given concentration and solvent flow rate. Such a balance was required to determine the solvent volumetric flow rate at system pressure and temperature. The oleic acid conversion was calculated using the initial oleic acid concentration and the concentration in the exit stream which comes to 6.70 ml/min giving a 15% conversion and hence the rate of reaction ($-V_{OA}$) is 1585 μmol/min/g_{cat}.

3.5. Experimental design

Response surface methodology (RSM) based on calibrating full quadratic models around the central composite rotatable design (CCRD) was adopted to develop a five factorial experimental design to optimise the reaction conditions for lipase mediate ethanolysis of oleic acid under SCCO₂. Similar statistical techniques [14] were adopted for the enzymatic synthesis of cocoa butter analog from camel hump fat in supercritical carbon dioxide. The experimental parameters, pressure of the reaction X_1 (bar); temperature of the reaction X_2 (°C); ethanol concentration, X_3 (M), enzyme concentration, X_4 (weight percentage of the reactants) and X_5 , residence time (min); were selected as the variables to maximize the response (conversion of FFA to esters). The experimental design generates using the RSM suggested 32 experiments with five variables at five levels (Table 1 – complete data not shown). A second order polynomial equation was developed to study the effects of variables on the esterification. The equation indicates the effect of variables in terms of linear, quadratic and cross-product terms.

$$Y = A_0 + \sum_{i=1}^N A_i X_i + \sum_{i=1}^N A_{ii} X_i^2 + \sum_{i=1}^{N-1} \sum_{j=i+1}^N A_{ij} X_i X_j \quad (2)$$

where Y is the conversion of esters (%), X_i is the variable, A_0 is the constant term, A_i is the coefficient of the linear terms, A_{ii} is the coefficient of the quadratic terms, A_{ij} is the coefficient of the cross-product terms and N is the number of variables. The coefficients of the equation were determined by using Design Expert 7.0 software. The analysis of variance ANOVA and the lack-of-fit for the final predictive equation were also studied. A graphical representation of Eq. (2) in the form of 3D plots was used to describe the individual and cumulative effect of the test variables on the response.

Table 1

Central composite rotatable second-order design, experimental data for five-level five factors response surface analysis.

	Pressure [bar]	Temperature [°C]	Ethanol conc. [M]	Enzyme conc. [wt%]	Residence time [min]	Predicted conversion [%]	Observed conversion [%]	Residual values
1	150	50	1.5	7.5	40	60.2	61.5	1.3
2	200	60	1.0	5.0	60	66.2	67.0	0.8
3	200	60	2.0	10.0	60	93.5	95.0	1.5
4	250	50	1.5	7.5	40	78.1	76.5	−1.6
5	150	50	1.5	7.5	40	60.2	60.0	−0.2
6	200	60	1.0	10.0	20	44.1	45.0	0.9
7	100	40	2.0	10.0	60	82.9	85.0	2.1
8	150	50	1.5	7.5	40	60.2	61.0	0.8
9	100	60	1.0	5.0	20	3.15	3.50	0.4
10	150	30	1.5	7.5	40	40.3	37.5	−2.8
11	200	40	1.0	10.0	60	88.1	90.0	1.9
12	150	50	1.5	2.5	40	44.1	43.5	−0.6
13	150	50	1.5	7.5	40	60.2	61.5	1.3
14	200	40	1.0	5.0	20	60.6	61.0	0.4
15	150	50	0.5	7.5	40	42.4	40.0	−2.4
16	150	50	1.5	7.5	40	60.2	60.0	−0.2
17	150	50	1.5	7.5	40	60.2	62.0	1.8
18	100	60	1.0	10.0	60	85.1	87.0	1.9
19	100	40	2.0	5.0	20	−0.6	0.0	0.6
20	150	70	1.5	7.5	40	44.5	43.0	−1.5
21	100	60	2.0	5.0	60	59.0	60.0	1.0
22	100	60	2.0	10.0	20	40.9	42.0	1.1
33 ^a	200	60	2.0	10.9	60	100	94.3	−5.7

^a Validation experiment at optimal conditions.

4. Results and discussion

4.1. Lipase mediated fatty acid esterification in batch mode

In order to understand the influence of process configuration a series of preliminary experiments were conducted in batch mode using hexane and supercritical CO₂ as the solvents. Fig. 4 shows that at a temperature of 55 °C using batch configuration the maximum 40% conversion of FFA to esters can be achieved in 24 h using 15 wt.% enzymes. Results clearly showed that the conversion increases with higher concentrations of enzymes and residence time up to 5 h, then remained constant. The supercritical CO₂ addition causes significant increase in conversion (up to 52% in 4 h at 55 °C using 10 wt.% enzyme) in comparison with use of hexane as a solvent which gave only 20%. This is due to the fact that ethanol and water are more soluble in SCCO₂ than in n-hexane [15,16]. A detailed comparison of both the solvents was conducted by Marty and Dumont [17,18] who found that the rate of reaction was influenced by the concentration of water and the composition of the reaction medium. Their findings did not conclusively show that a supercritical fluid has confirmed advantages over conventional solvents for enzymatic reactions. This study has shown that supercritical CO₂ is a better medium compared to organic solvents (e.g., hexane). The findings are supported by Chi [16] using lipase in SCCO₂ at 50 °C at 294 bar in batch reactor.

4.2. Optimisation of reaction parameters for continuous flow fatty acid esterification

From a process development perspective the yields and associated residency time are limiting. To address this problem a test rig (Fig. 1) was designed and made that would support continuous flow lipase mediated esterification using SCCO₂ as the solvent.

The objective of the research was to develop a clear understanding of the impact that the operating parameters have on the efficiency of lipase mediated fatty acid esterification, in order to optimise the reaction under continuous flow SCCO₂ conditions. Table 1 illustrates the combination of parameters evaluated as dictated by the experimental plan generated using RSM. The empirical results deviated from the predicted rate derived from the RSM

model within a range of 5% which is considered an acceptable technical error (Table 2). The final estimated response model equations were obtained as below (in terms of original factors):

$$\begin{aligned} \text{Conversion [\%]} = & -205.501 + 1.375X_1 + 3.9 - X_2 + 32.910X_3 \\ & - 5.051X_4 - 0.189X_5 - 0.009X_1X_2 - 0.193X_1X_3 - 0.039X_1X_4 \\ & - 0.002X_1X_5 + 0.443X_2X_3 + 103X_2X_4 + 0.015X_2X_5 + 2.025X_3X_4 \\ & + 0.353X_3X_5 + 0.073X_4X_5 - 0.00002X_1^2 - 0.044X_2^2 \\ & - 18.768X_3^2 + 0.309X_4^2 - 0.006X_5^2 \end{aligned} \quad (3)$$

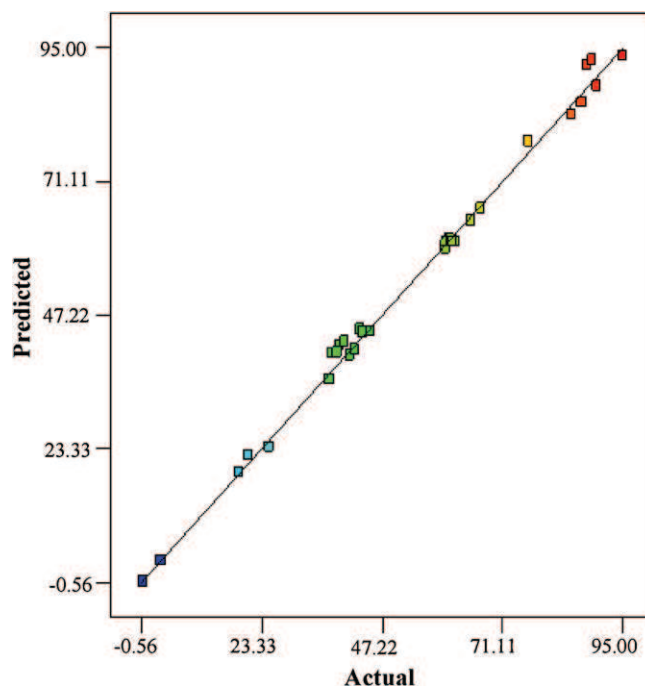
**Fig. 2.** Plot of predicted response values versus the actual response values.

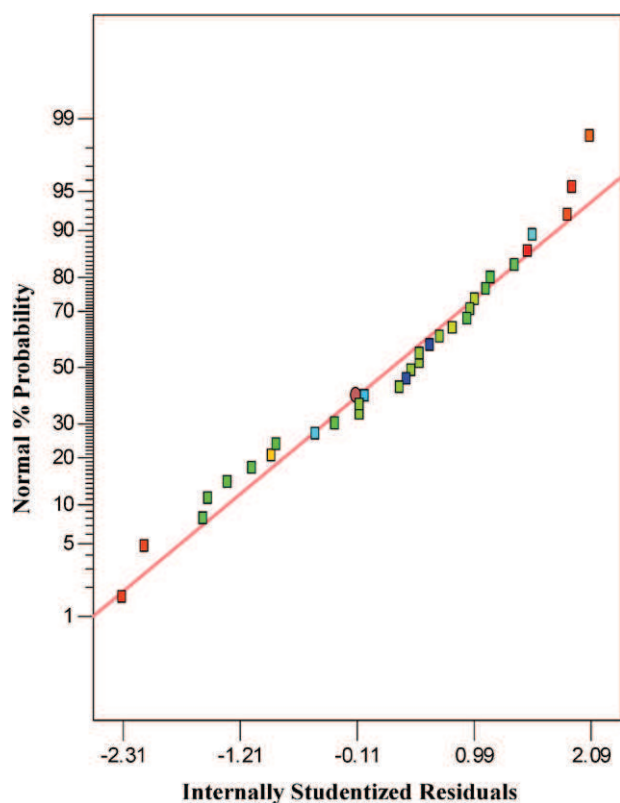
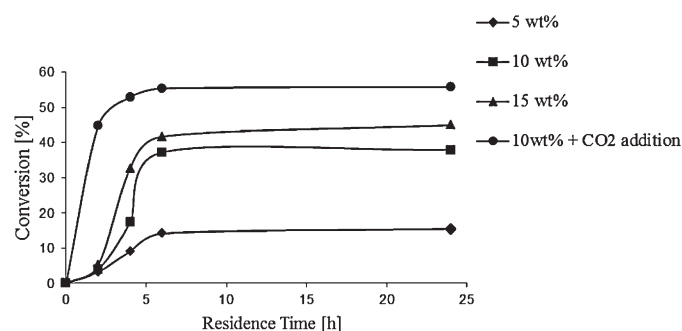
Table 2

Analysis of variance (ANOVA) for the fit of the experimental data to response surface quadratic model.

	Sum of squares	Degree of freedom [df]	Mean squares	F value	P value
Model	18946.97	20	947.35	113.86	<0.0001
X1-pressure	1989.26	1	1989.26	239.08	<0.0001
X2-temperature	27.09	1	27.09	3.26	0.0986
X3-ethanol conc.	5.51	1	5.51	0.66	0.4330
X4-enzyme conc.	3420.09	1	3420.09	411.05	<0.0001
X5-residence time	9868.23	1	9868.23	1186.04	<0.0001
X1X2	365.77	1	365.77	43.96	<0.0001
X1X3	375.39	1	375.39	45.12	<0.0001
X1X4	395.02	1	395.02	47.48	<0.0001
X1X5	141.02	1	141.02	16.95	0.0017
X2X3	78.77	1	78.77	9.47	0.0105
X2X4	107.64	1	107.64	12.94	0.0042
X2X5	153.14	1	153.14	18.41	0.0013
X3X4	102.52	1	102.52	12.32	0.0049
X3X5	199.52	1	199.52	23.98	0.0005
X4X5	213.89	1	213.89	25.71	0.0004
X1 ²	0.13	1	0.13	0.02	0.9016
X2 ²	582.14	1	582.14	69.97	<0.0001
X3 ²	649.51	1	649.51	78.06	<0.0001
X4 ²	110.21	1	110.21	13.25	0.0039
X5 ²	140.73	1	140.73	16.91	0.0017
Residual	91.52	11	8.32		
Lack of fit	88.02	6	14.67	20.96	0.0021
Pure error	3.50	5	0.70		
Total	19038.49	31			
CV = 5.41%	$R^2 = 0.995$	$R^2_{Adj} = 0.986$	Predicted $R^2 = 0.883$		

The results of ANOVA for fitting the second order response surface model by a least square method are presented in Table 2. The high F value (F model = 113.85) with very low probability value ($P < 0.0001$) indicates the high significance of the fitted model. The goodness of fit of the model was evaluated by the adjusted coefficient of determination (R^2). The significance of all coefficients was established by P -values shown in Table 2. The smaller the P value

for a parameter the more significant a parameter is, this reflects the relative importance of the term attached to that parameter [19]. In linear terms pressure, enzyme concentration and residence time, and in quadratic terms, squared reaction temperature and solvent concentration (P -value = <0.0001) made the most significant contributions to the fitted model. Furthermore, all first order interaction effects of the five predictors were statistically significant, with the greatest significance for the interaction of pressure \times temperature, pressure \times solvent concentration and pressure \times enzyme concentration (P -value = <0.0001). At the same time, the low value of the coefficient of variation ($CV = 5.41\%$) indicates that results of the fitted model are reliable. A high value of coefficient of determination ($R^2 = 0.995$) is an indication of reasonable precision of the model fitted. The value of the adjusted coefficient of determination ($R^2_{Adj} = 0.986$) is also very high, supporting the significance of the model. Fig. 2 shows that the values of the response predicted from the empirical model are in agreement with the observed values over the selected range of the operating variables with a reasonable high value of coefficient of determination ($R^2 = 0.995$). As can be seen in Fig. 2, most of the points are located in the range 50–95%. Only a couple of points are located below 50%. The residuals must be normally distributed for the results of ANOVA to be valid [20]. Normal probability of the residuals was checked by a normal probability plot

**Fig. 3.** Normal probability plot of residuals.**Fig. 4.** Effect of enzyme concentration and addition of CO_2 on the rate of esterification reaction.

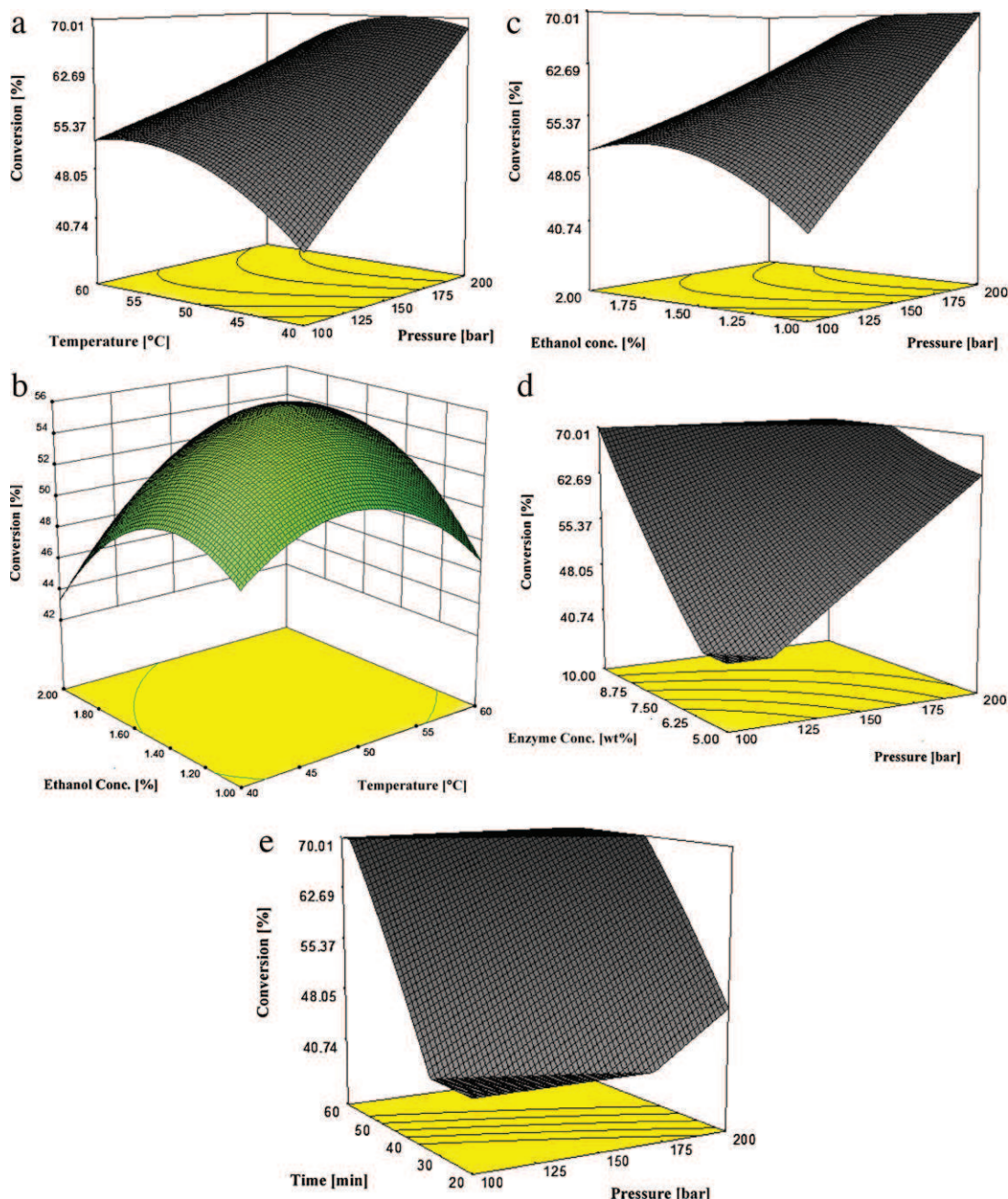


Fig. 5. (a) Plot shows the mutual effect of temperature and pressure at 40 min, 7.50 wt.% enzyme and 1.50 ethanol concentration on the conversion of esters. (b) Plot shows the mutual effect of temperature and ethanol concentration at 40 min, 7.50 wt.% enzyme and pressure of 150 bar on the conversion of esters. (c) Plot shows the mutual effect of ethanol concentration and pressure at 50 °C, 40 min and 7.50 wt.% enzyme concentration on the conversion of esters. (d) Plot shows the mutual effect of enzyme concentration and pressure at 50 °C, 40 min and 1.50 ethanol concentration on the conversion of esters. (e) Plot shows the mutual effect of time and pressure at 50 °C, 7.50 wt.% enzyme and 1.50 ethanol concentration on the conversion of esters.

(Fig. 3). The plot approximates a straight line, confirming normality of the data. As the fitted model Eq. (3) provides a good approximation to the experimental results, the model was employed to find the values of the process variables for maximum yield of the fatty acid ester.

3D surfaces plots, which are the graphical representation of the relationship between the responses and process parameters, are presented in Fig. 5(a)–(e). The plots were made by taking an infinite number of combinations of the values of the two test variables at a time and keeping the values of the remaining two test variables constant. The plots are useful for understanding the interaction of two test variables and determining their optimum levels by holding other test variables constant.

The response surface Eq. (3) was optimized to identify the optimum process conditions for the maximum conversion of oleic acid. The process conditions that predicted a 100% conversion were: pressure 200 bar, temperature 60 °C, ethanol concentration 2 M, enzyme 11 wt.% and residence time of 60 min. Experiments conducted under these conditions gave a conversion of 94%. The difference could be attributed to the noise generated in the model equation.

4.3. Effect of pressure on the fatty acid ester yield

The reaction pressure primarily influences enzyme activity, whereas the effect on enzyme stability is less pronounced. An

increase in pressure of the supercritical fluid normally enhances the conversion rate due to the increased analyte solubility [21,22], however, at some point, the enzyme activity starts decreasing with increasing pressure [23,24]. This has been attributed to the lower mass transfer rates of reactants with increase in SC-CO₂ density [24]. The 3D plots Fig. 5(a)–(e) indicate that, at a pressure of around 200 bar the maximum ester yield (70%) was obtained with the other variables at their optimum levels. As pressure increased however, the conversion of FFA into esters did not vary considerably under the conditions studied. The pressure range of 100–200 bar was studied previously by Knez [25], they reported the pressure of 150 bar is optimum, due to the unchanged activity of the enzyme, for the esterification of oleic acid with oleyl alcohol using *Rhizomucor miehei* lipase with SCCO₂. A change in pressure can affect the density dependent physical properties (partition coefficient, dielectric constant and Hildebrandt solubility parameter) of SCCO₂ which can indirectly modulate enzyme activity, specificity and stability [26].

Fig. 5(a)–(e) shows that the conversion of FAEE did not increase significantly with an increase in pressure, in the range studied (100–200 bar). This could be as a result of the pressure-induced denaturation of the enzyme due to conformational changes, although an increase in pressure increased the substrate solubility at a certain reaction temperature, and facilitated the reaction rate while decreasing the partition of substrates between the immobilized enzyme and supercritical solvent phases. In addition, the increase in pressure also results in a decrease in the diffusion due to the increases in density and viscosity of SCCO₂. Higher temperatures also contribute to the differential partition of the substrates in the SCCO₂ phase and the enzyme phase, along with increased pressure, as found by Shishikura [27]. Fig. 5(a) shows at lower pressures at 40 °C the conversion is 20%. By increasing the temperature to 60 °C the conversion increases by up to 50% this effect is not pronounced by higher pressure as only 60% conversion occurs at 60 °C with a pressure of 200 bar, at higher temperatures the risk of thermally denaturing the enzyme is increased [8].

4.4. Effect of temperature on the fatty acid ester yield

Temperature significantly affected enzyme catalysis in SCCO₂. Fig. 5(a) shows that the maximum ester conversion of 70% was obtained at 60 °C with other variables at different levels. The conversion of esters decreased at temperatures below and above this value. Temperature influences the 3D structure and hence stability of the enzyme. It also affects the partition of substrates between the SCCO₂ phase and enzyme phase [28]. Fig. 5(b) shows that at higher temperatures there is a lower rate of conversion due to the lower availability of the alcohol substrate (ethanol) – also reported by Ramamurthi [29]. Increased concentrations of ethanol can enhance the deactivation effect of temperature on enzyme activity as reported by Knez and Ergun [30,31]. When the temperature is at 60 °C, an ethanol concentration of 2 M gives 55% conversion of FAEE as observed in Fig. 5(b). The reaction temperature affects both the activity and stability of the enzyme. Increasing temperature increases diffusion rates, thereby resulting in faster reactions, since the reaction rate is, in most cases limited by slow diffusion of larger molecules into the porous enzyme support material. However as mentioned in Section 4.3 higher temperatures increase the risk of thermally denaturing the enzyme. There is usually an optimal reaction temperature, which is dependent on type of enzyme, support material, immobilization technique and reaction medium.

4.5. Effect of ethanol and enzyme concentration

Fig. 5(c) shows the effect of ethanol concentration with other reaction parameters, at varied levels, on the conversion of FAEE. At an ethanol concentration of 2 M, increased enzyme concentration, lower temperatures and higher residence time were found to give the maximum conversion of FAEE. The decreased ethanol concentration eventually causes the incomplete conversion of the FFA into their esters. On the other hand, ethanol concentration above optimum levels was detrimental to the action of the enzyme with the interaction of other reaction parameters particularly the high temperature levels. Alcohols as substrates alone, at higher concentrations have the ability to inhibit the catalytic activity of lipases [32]. Fig. 5(d) shows the effect of enzyme concentration, with the interaction of other reaction parameters, on the conversion of FAEE. Its effect was found to be considerable in obtaining maximum conversion of FAEE. The existing results [33] are comparable to the results in this study, i.e., the effect of enzyme concentration on the conversion of FAEE at increased enzyme concentrations gives increased yield of the FAEE in the range (5–15%). The saturation effect of enzymes on the conversion of FAEE was not observed within the experimental range studied.

Higher concentrations of enzymes are required to achieve increased conversion at lower pressures. At high levels of pressure, 60–70% conversion of the FAEE can be obtained with lower enzyme concentrations, but maximum yield cannot be achieved. Hence, high level of enzyme concentration (10 wt.%) is necessary to obtain the maximum conversion of FAEE, with all other reaction parameters at their optimum levels. The other reaction parameters should be maintained at optimum level with higher enzyme concentration as they will inhibit the catalytic action of enzymes at below and above optimum levels.

4.6. Effect of residence time

The 3D plot (Fig. 5(e)) shows that, under optimum reaction conditions, there was a steady increase in the conversion of FAEE with increased residence time. Maximum conversion of 95% of FAEE is possible with 60 min of residence time with SCCO₂. At higher temperatures, it is possible to achieve high conversion, in less residence time, as the rate of the reaction seems to be faster, but maximum conversion cannot be obtained. Even the increase in pressure does not show any significant improvement in the conversion with increased residence time. In turn the increased pressure decreased the conversion of FAEE. The reason for this observed effect was explained in Section 4.3.

5. Conclusion

All reaction variables have their own effect on the conversion of FAEE, this has been shown by the optimal ethyl esterification of FFA by CCRD design under SCCO₂ in association with other parameters. The mutual effect of temperature and ethanol concentration on the conversion of FAEE is highly pronounced. The effect of pressure on the stability of enzymes is usually quite small. The predicted model fits well with the experimental results. The esterification process of FFA, as part of the biomodification process under SCCO₂ in a continuous configuration is found to have potential compared to the same with the shake-flask method as it shows faster reaction. That is, under SCCO₂ esterification is achieved in 60 min to the maximum of 95%, however with the shake-flask method it took 6 h to obtain a yield of 55%.

Acknowledgements

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Continuous Flow Hydrolysis of Sunflower Oil for Biodiesel

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Abstract *Free fatty acids are an important intermediate for several industrial applications, particularly for production of biodiesel via methanolysis. The use of subcritical water as both solvent and reactant for the hydrolysis of vegetable oil to generate fatty acids has recently been proven to be a successful medium for hydrolysis without employing acid or alkali catalysts, while allowing for a simple process and high yield. Continuous flow hydrolysis of sunflower oil in subcritical water to obtain FFA was investigated in a tubular reactor at 10 to 20 MPa, 270 to 350°C and water/oil ratios of 80:20 and 50:50 v:v%. The rate of the hydrolysis was enhanced significantly by increasing reaction temperature and decreasing the water/oil ratio.*

Keywords free fatty acids, hydrolysis, subcritical water, sunflower oil

1. Introduction

Biodiesel is comprised of esters of short chain alcohols made from renewable biological sources, such as vegetable oil and animal fats, and can be used as substitute diesel fuel or more commonly in fuel blends (Lopez et al., 2005). In comparison to petroleum-derived diesel, pure biodiesel (B100) releases about 90% of the energy, and hence, its engine performance is approximately the same in terms of engine torque and horsepower.

Biodiesel can be produced through transesterification of triglycerides with methanol. Most transesterification methods use alkali catalysts, which lead to the production of undesirable saponified products as the free fatty acids (FFA) react with the catalyst. These require sophisticated purification steps and result in a lower yield of the desirable fatty acid methyl esters (FAME; biodiesel). Some researchers (Granados et al., 2007; Izci and Bodur, 2007; Vicente et al., 2007) have proposed an acid-catalyzed method coupled with an alkali catalyst process to overcome these disadvantages, but it is advantageous to develop an entirely catalyst-free process to eliminate downstream separation of the catalyst and improve process economics. Saka and Dadan have developed a catalyst-free, two step process for high-quality biodiesel production from oils and fats. The process first uses subcritical water to hydrolyze triglycerides to fatty acids (FA) followed by a methyl esterification step to FAME using supercritical methanol without any catalyst. In

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addition to the use in biodiesel production, FA and glycerol are important raw materials for soap production, synthetic detergents, greases, and cosmetics (Lawrence, 1954).

Water in the sub or supercritical state provides unique properties over water at room temperature and at ambient pressure. The two distinct advantages of subcritical water are its relatively low dielectric constant and large ion products. The dielectric constant of subcritical water (10 to 20 MPa, 270 to 350°C) is comparable to that of acetone or methanol at ambient conditions and can be adjusted from a value of 80 to 5 at its critical point, which makes it a good solvent for organic compounds (Holliday et al., 1997). Many studies have been undertaken to explore the use of water near its critical condition (374°C, 22.1 MPa) to eliminate organic solvents in synthesis reactions (Ackelsberg, 1958; Lascaray, 1952; Partil et al., 1988; Pinto and Lancas, 2006). In the hydrolysis of triglycerides, subcritical water has been shown to act as both a solvent and a reagent (Partil et al., 1988; Pinto and Lancas, 2006).

The hydrolysis reaction shown in Figure 1 occurs as three stepwise reactions: (1) triglyceride (TG) is hydrolyzed to diglycerides (DG), (2) DG is hydrolyzed to monoglycerides (MG), (3) MG is hydrolyzed to glycerol, and in each step there is a production of a FA. This reaction is a homogenous first order reversible reaction in the oily phase (Ackelsberg, 1958). It has been shown that FA itself can act as an acid catalyst in the hydrolysis reaction of oil in subcritical water and can achieve up to 90 wt% FA without employing a catalyst (Minami and Saka, 2006). This non-catalytic reaction prevents the need to remove the catalyst from the final product which is technically difficult and decreases the production cost of biodiesel (Demirbas, 2003).

Biodiesel can be prepared from numerous sources of vegetable oils, animal fats, and waste greases. Researchers have reported the use of linseed oil, castor oil rapeseed (Varma and Madras, 2007), and sunflower oil (Granados et al., 2007) as oil sources; however, the data available for sunflower oil is focused on catalytic transesterification (as is the majority of the work for other oils) and data for catalyst-free subcritical water processing are unreported. This work investigates continuous flow hydrolysis of sunflower oil using subcritical water as a route to FA for biodiesel production for the first time. A non-catalytic continuous flow process was investigated between 10 to 20 MPa, 270 to 350°C for two volume percent mixtures of water and oil. The factors affecting the yield and rate of reaction were studied.

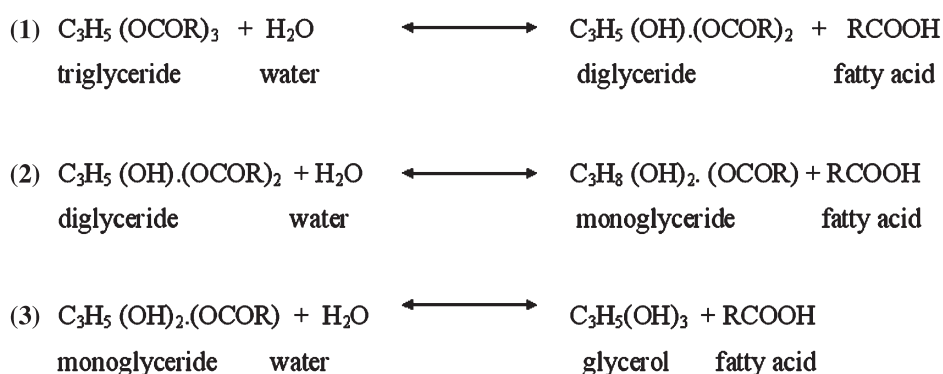


Figure 1. Hydrolysis reaction of vegetable oil.

2. Material and Methods

Sunflower oil was purchased from a leading supermarket chain based in the UK. The sunflower oil consisted of about 77 wt% triglycerides (TG); 20% diglycerides (DG); 2.5% monoglycerides (MG); and only 0.3 to 0.5% FFA, as analyzed by gas chromatography (GC). Distilled and deionized water was used to prevent scaling of the walls of the tube reactor and ancillary piping at the reaction conditions. Figure 2 shows the laboratory-scale setup of the equipment used for the hydrolysis of sunflower oil under continuous flow conditions. In this system, the oil and water were separately fed by two HPLC pumps from the reservoir columns into the coiled tubular reactor through a mixer device. The dosage was adjusted to give the desired volumetric water/oil ratios. As the reaction conditions were not in the supercritical water region, the reactor was made from stainless steel tubing, which had an internal volume of 60 ml. The reactor was housed in an electrical furnace and the pressure in the reactor was controlled by a back pressure regulator valve. In this way, the temperature and the pressure inside the reactor coil were continuously maintained at the desired operational conditions. The conditions investigated ranged between 250 to 350°C and 10 to 20 MPa. The reaction time t , was calculated by dividing the volume of the reactor by the volumetric flow rate of the oil and water under the experimental conditions using Eq. (1) as follows (Minami and Saka, 2006):

$$t = \frac{V}{F_w \frac{\rho_w}{\rho'_w} + F_o \frac{\rho_o}{\rho'_o}} \quad (1)$$

where V is the volume of the reactor; F_w is the set flow-rate of water (ml/min); ρ_w and ρ'_w are the densities (g/ml) of the water at the normal ambient condition and reaction condition, respectively; F_o is the set flow-rate of oil (ml/min); and ρ_o and ρ'_o are the densities (g/ml) of the sunflower oil at the normal ambient conditions and reaction conditions, respectively, and is assumed to be equal at the reaction conditions.

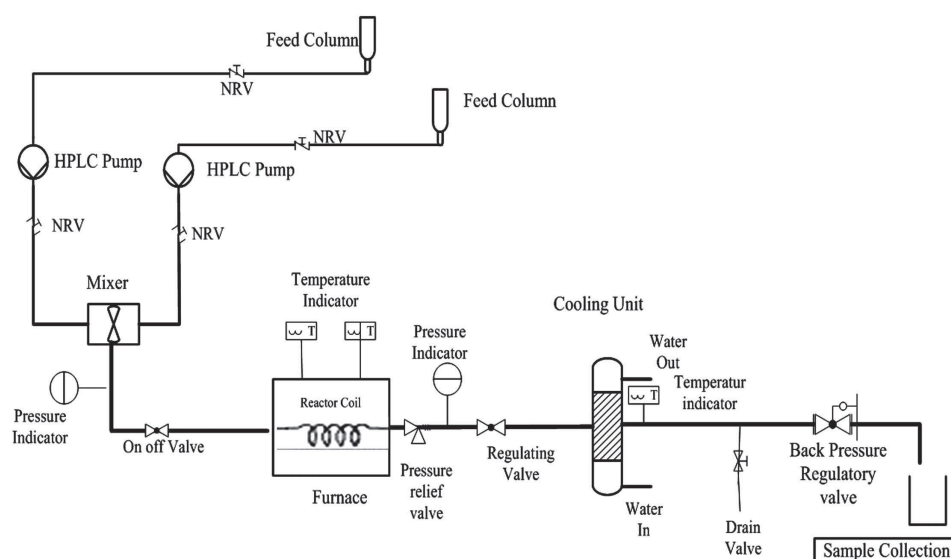


Figure 2. Subcritical water continuous flow hydrolysis rig.

Upon leaving the reactor, the products were cooled in a cooling unit to ambient, and samples were collected at regular time intervals. The hydrolysis products were gravity separated into two portions: the upper portion being mainly FA, unreacted TG and intermediate compounds (DG and MG), and the lower portion, glycerol. The samples were analysed by two methods (detailed below) to determine the total yield of FA.

2.1. Fatty Acid Analysis by GC

The concentration of fatty acid in the product was determined by a gas chromatograph (HP 6850A, Agilent Technologies, USA) equipped with capillary column DB-5 (length 30 m \times diameter 0.25 mm \times film thickness 0.1 micrometer; J&W Scientific, USA) and a flame ionization detector (340°C). Helium was used as the carrier gas at a constant flow (2 ml/min) with a split injector (340°C, 1:50). The oven temperature program was 100°C for 3 min, 10°C/min to 150°C, 5°C/min to 250°C, 10°C/min to 350°C, with 15-min intervals on hold. The samples were derivatized to increase the volatility of the fatty acid components. These were prepared by adding 1 ml chloroform, 10–30 mg of heptadecanoic acid as the internal standard and 1 ml hexamethyldisilazane to 100–200 mg of the sample and heating for 50 min at 70°C. After reaction, the solution was allowed to cool to room temperature before injection of the silylated samples into the gas chromatograph. A fatty acid component was identified from reference standards and its mass was calculated from a predetermined peak area response factor of the internal standard (heptadecanoic acid). The total yield of free fatty acids was determined by the addition of all fatty acid masses. Injection errors were minimized by comparing the peak area of the internal standard to a predetermined set value and applying any necessary corrections.

2.2. Fatty Acid Determination by Titration

An official method proposed by the American Oil Chemist Society (Ca 5a-40) was used to determine the percentage of fatty acid in the collected samples. This method can be applied to all crude and refined vegetable oils. The procedure may be described briefly as follows: 1–2 g of the sample was weighed into a conical flask and 50–100 ml ethyl alcohol (97%) was added to give a definite and sharp titration end point. Phenolphthalein indicator was then added. The mixture was titrated against sodium hydroxide (0.25 N) until a permanent pink color persisted for at least 30 s. The weight percentage of FA was calculated on an oleic acid basis using Eq. (2). Most of the samples were titrated in duplicate with a variance of <0.5%, which is shown as follows:

$$\text{Free fatty acids as oleic, \%} = \frac{\text{ml of alkali} \times N(\text{conc. of NaOH}) \times 28.2}{\text{wt. of sample}} \quad (2)$$

3. Results and Discussion

Fatty acid analysis: The major fatty acids distribution in the sunflower oil were identified by GC to be palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2), which contributed to 97% of the total FA content. The concentration distribution of fatty acids measured by the silylation/GC and titration methods is shown in Figure 3. Both methods compare well and give a strong correlation ($R^2 = 0.98$), though the GC tends to give higher values, in particular in the 30 to 80% range. Both techniques

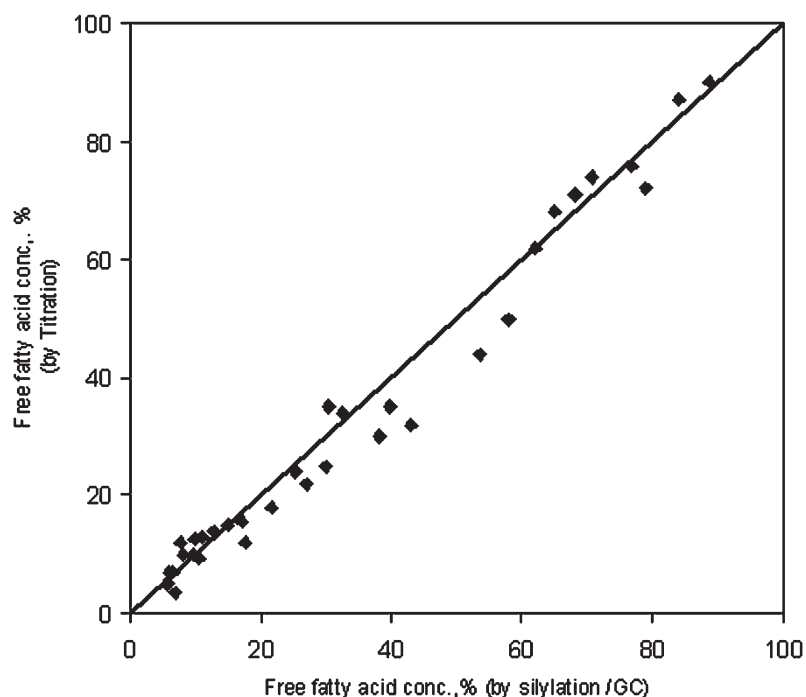


Figure 3. Comparison analysis of fatty acid obtained by titration and GC.

are acceptable for determining FA content following the subcritical water hydrolysis and certify the results with an appropriate assurance; however, the silylation/GC method enables the determination of the concentration of such FFA component, while the titration method only measures the total FFA. Analysis of the four main fatty acids that were obtained at 300°C, 15 MPa and 50/50 water/oil ratio are shown in Figure 4. There is an increase in total FA yield as the reaction has progressed. Selectivity towards a particular FA component does not vary with reaction time. The hydrolysis tests were repeated for each particular experimental condition. The greatest standard deviation was observed for the test at 300°C, 20 MPa, and 50/50 water/oil ratio, which gave a value of 5.4% FA yield.

3.1. The Effect of Pressure on FA Yield

The effect of pressure on FA yield was investigated between 10–20 MPa at 300°C and a water/oil ratio of 50/50 vol%. It can be seen in Figure 5 that as pressure was increased from 10 to 15 MPa there was a slight increase in the yield of FA. Above 15 MPa the pressure has less effect in the hydrolysis reaction and does not cause an obvious improvement in FA yield.

3.2. Effect of Temperature on FA Yield

The effect of temperature on the yield of FA was investigated between 270 and 350°C at 20 MPa using 50/50 water to oil ratio. From Figure 6, it can be seen that the yield

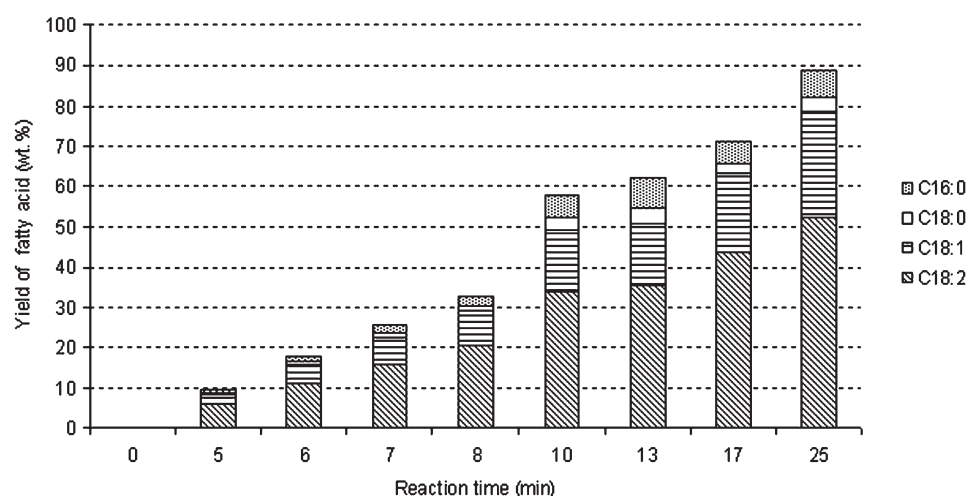


Figure 4. Conversion of four main fatty acids at 15 MPa, 300°C, and water/oil ratio 50/50 v/v%.

of FA at 270°C very slowly increases in the early stages of the reaction. As water and oil are insoluble at temperatures below 250°C, reactions in these conditions are controlled by transport properties. Increasing the temperature increases the oil solubility in water and the speed of the reaction accelerates quickly (Pinto and Lencas, 2006) as is evident here. Ackelsberg (1958) reports that an increase of 10°C in the temperature increases the rate of hydrolysis by a factor of 1.2 to 1.5. A FA yield greater than 90% can be achieved in 8 min at 350°C without catalyst addition; this is due to the chemical nature of the fatty acids acting as an acid catalyst in the hydrolysis reaction (Minami and Saka, 2006) and an increase of the ionic product of water. Above 300°C

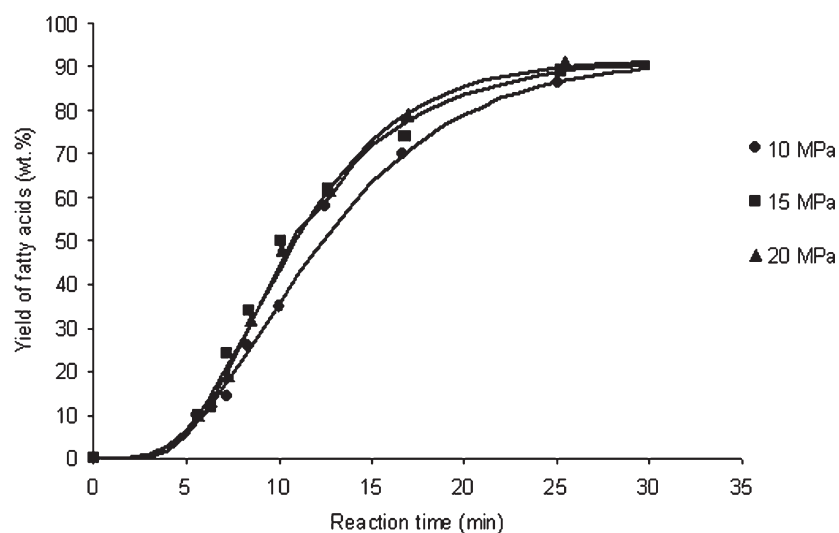


Figure 5. Effect of pressure in the total fatty acids yield at 300°C and water/oil ratio 50/50 v/v%.

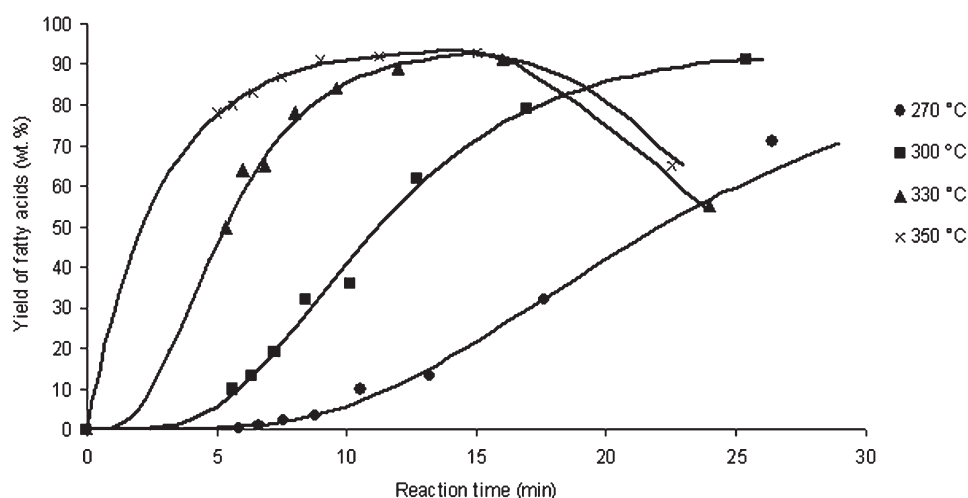


Figure 6. Effect of temperature in the total of fatty acids yield at 20 MPa and water/oil ratio 50/50 v/v%.

the hydrolysis reaction reaches equilibrium at around 90 wt% of FA after 25 min. At 330 and 350°C at reaction times greater than 16 min, the yield of FA decreased. This is either due to polymerization of the FA with the remaining unreacted TG or decomposition and pyrolysis reactions (Holliday et al., 1997). Generally, higher temperatures lead to high FA yields and other researchers (Weatherley and Rooney, 2008) have reported enhanced rates of reaction and FA yields using a high voltage electrical field, which can be considered a temperature effect due to localized heating. The rate of enzymatic hydrolysis of sunflower oil increased as the magnitude of the applied electrical field was increased.

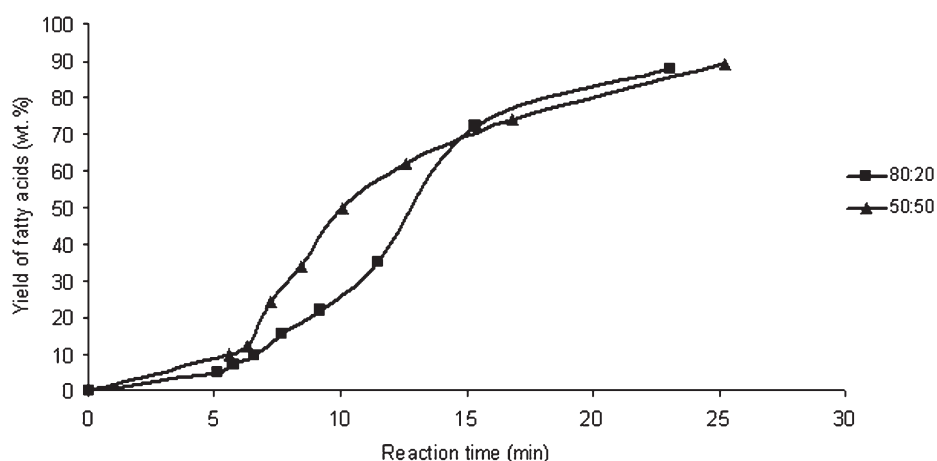


Figure 7. Effect of water/oil ratio in fatty acid yields at 15 MPa and 300°C. (■, water/oil 80/20 (v/v%); ▲, water/oil 50/50 (v/v%)).

3.3. Effect of Water Oil Ratio on FA Yield

The effect on water/oil ratio at 15 MPa and 300°C is shown in Figure 7. In a similar manner to an increase in temperature, a decrease in the water/oil ratio results in a higher FA yield and in a shorter reaction time. At a reaction time of 11 min, a yield of 55% FA was obtained for a water/oil ratio of 50/50 v/v%, compared to 35% FA when the water/oil ratio was diluted to 80/20 v/v% in the dilute system. The amount of FA available prior to subcritical water treatment would have been less and therefore its catalytic contribution would be less. This indicates that the ratio of water/oil is an essential parameter in the degree of hydrolysis. If the reaction time is sufficient, the dilute system eventually gives higher FA yield due to increased collisions. As more FA is produced, there is a shift in the equilibrium to the right side and the reaction time decreases.

4. Conclusion

The hydrolysis of sunflower oil triglycerides by subcritical water is a promising method for production of a high yield of FA. The continuous process offers the advantage of large-scale production with limited labor requirements, while maintaining product quality. Hydrolysis reaction with a high yield of up to 90% FA within 7 to 15 min at 350°C, 20 MPa, and water/oil ratio 50/50 v/v% can be achieved without the need for catalysts. It has been found that the rate of hydrolysis is strongly affected by temperature, reaction time, and the water/oil ratio; however, the reaction pressure has a minor effect on the degree of hydrolysis. In order to achieve quantitative hydrolysis there is a need to extend the reaction time, but care should be taken to avoid the thermal degradation of the products. The extension of the continuous flow technique to FAME formation is currently being investigated.

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HYDROLYSIS OF BIOMASS DERIVED COMPONENTS; LIPIDS AND CARBOHYDRATES

Article Type: Full Length Article

Keywords: Hydrolysis; Sub-critical water; Biomass; Lipids; Carbohydrates;
Response surface methodology (RSM)

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Abstract: Sub-critical water (SCW) has successfully been used as a solvent for extraction of range of compounds and as a reaction medium. Therefore to demonstrate the versatility of SCW as a generic medium to support hydrolysis; rice bran oil, beef tallow, sunflower oil (lipid substrates) and rice bran (carbohydrates) were chosen as model substrates as they possess very different physio-chemically properties therefore allowing the impact of the ion product and dielectric constant of water to be evaluated. Results from both batch and continuous processing with SCW indicate that both carbohydrate polymers and triacylglycerols can be efficiently (> 90%) hydrolysed within 60 min. The reaction kinetics and response surface methodologies (RSM) were used to assist with modelling the sub-critical water hydrolysis of sunflower oil and successfully validated, thereby confirming the utility of the RSM as a tool to assist in process development.

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Dear Editor,

Please find attached a manuscript entitled '**Evaluation and modelling of continuous flow subcritical water hydrolysis of biomass derived components; lipids and carbohydrates**' for consideration for publication in the Chemical Engineering Research and Design.

The manuscript describes work undertaken to evaluate the utility of sub-critical water as an environmentally benign solvent to support the processing of lipids and carbohydrates under a continue flow configuration. Moreover the work successfully validates kinetic and response surface models as potential tools that assist process optimisation. To the knowledge of the authors, this is the first example where the utility of sub critical water has been evaluated to support the processing of both lipid and carbohydrate in view of developing integrated bio refining. This work also addresses the growing concerns relating to biorefinery concept (in particular, bioethanol production) as it suggests a benign route for the full utilization of all forms of biomass.

Based on this, we would appreciate your recommendation for the publication of this manuscript.

Thanks in advance,

Yours sincerely,

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- 1- SCW is used as a catalytic medium for processing lipids and carbohydrates.
- 2- To validate kinetic and RSM as potential tools that assist process optimisation.
- 3- Carbohydrate polymers and TAGs can be efficiently (>90%) hydrolysed within 60 min.

**Evaluation and modeling of continuous flow sub-critical water hydrolysis of
biomass derived components; lipids and carbohydrates**

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1 **Abstract**

2 Sub-critical water (SCW) has successfully been used as a solvent for extraction of range of
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4 generic medium to support hydrolysis; rice bran oil, beef tallow, sunflower oil (lipid substrates)
5 and rice bran (carbohydrates) were chosen as model substrates as they possess very different
6 physio-chemically properties therefore allowing the impact of the ion product and dielectric
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13 **Keywords: Hydrolysis; Sub-critical water; Biomass; Lipids; Carbohydrates; Response**
14 **surface methodology (RSM)**

15

1. Introduction

The transition to a Knowledge-Based Bio-Economy (KBBE) is the vision within the European Union, US and other developed countries. Supporting the goal of achieving biobased economic development is the increased utilisation of industrial biotechnology which will foster more environmentally-benign manufacturing and chemical processes.

Coupling industrial biotechnology with the enhanced use of renewable and sustainable biomass enables the production of 'green' sustainable industrial feed stocks, thereby reducing our current dependence on fossil fuels. Due to the complexity of biomass and the fact that biomass is most often in a solid form, there is a need for a sustainable and environmentally-friendly pre-treatment process technology that will enable any biomass or organic waste to be fully utilised without creating waste streams associated with the process.

During the past decade, critical fluids have been recognised as environmentally-benign, 'green' solvents in part due to their unique physical and chemical properties(King and Srinivas, 2009). Chemists and engineers have developed an increased interest in sub and supercritical water (SCW) as they have proved to be versatile mediums for supporting both extraction and reactions. Water is a cheap, non-toxic, non-combustible and in fact water may be the most benign alternative to organic solvents depending upon the temperature range over which it is applied and the thermal ability of the solutes being extracted or reacted. Sub-critical water is hot water maintained between 100 °C and 374 °C (critical temperature) and below 22 MPa, its critical pressure. Many studies have been conducted to explore the use of water under sub- and supercritical conditions to promote organic reactions⁴. Sub-critical water has successfully been used as a solvent for the extraction of numerous compounds(Lanças, 2003), in part due the fact that the dielectric constant of water can be efficiently modified through modulation of pressure and temperature. Moreover, the ion product constant for sub critical water is in the order of 10^{-11} , which is three orders of magnitude larger than that exhibited by ambient water(Clifford, 1998; Savage, 1999). Due to the variation of

the ion product and its acidic nature, subcritical water can also be used to hydrolyse naturally occurring compounds such as triacylglycerides (TAG) and polysaccharides (King, 2004; Moreschi et al., 2004).

Therefore given the complex interplay between extraction (solubility) and hydrolytic reactions and the emerging potential of SCW as a versatile environmentally-benign solvent, our research objectives were to evaluate and model batch and continuous flow SCW hydrolysis of a range of biomass types. Biomass is principally, composed of four major chemical families, two of which are lipids and carbohydrates. Therefore to demonstrate the versatility of SCW, rice bran oil, beef tallow, sunflower oil (lipids) and rice bran (high in carbohydrate content) were used as substrates to illustrate the generic applicability of sub-critical water as a hydrolytic medium.

To investigate the impact of multiple processing parameters on the efficacy of sub-critical water mediated biomass hydrolysis, response surface methodologies (Montgomery, 1995) were adopted and evaluated as a potential tool to assist with the optimisation and modelling of the sub-critical water hydrolysis of sunflower oil and hydrolysis of rice bran, along with the determination of the resultant hydrolysis kinetics.

2. Materials

Rice bran was provided by Euryza GmbH (Hamburg, Germany). Prior to the hydrolysis experiments, the rice bran was milled and sieved to recover a particle fraction of less than 180 μm . The milled and sieved rice bran was then pelleted to a mean diameter of 0.50 mm by using a drum agglomerator (Eirich, Hardheim, Germany). The rice bran pellets were then defatted using supercritical carbon dioxide and the rice bran oil extracted at 30 MPa, temperature 60 $^{\circ}\text{C}$, for 3 h, at a solids/feed (S/F) ratio = 22 kg h^{-1} prior to hydrolysis (Danielski et al., 2005). Beef tallow (grade 6) was provided by Pointins and Sons (Cheddleton UK). Refined food grade Sunflower oil was purchased from Sainsburys (UK).

The reagents used for analysis were analytical grade, ethanol and sodium hydroxide (Fisher Scientific, UK), phenolphthalein (Hopkin and Williams Ltd. Essex, UK) and diethyl ether (Carl Roth GmbH, Karlsruhe, Germany). Carbon dioxide liquid withdrawal (5 MPa @15 °C) was used in the experiments from (BOC, UK)

3. Methods

3.1 Continuous flow sub critical water hydrolysis of lignocellulosic fraction of rice bran

Sub-critical water mediate hydrolysis of rice bran was conducted in the continuous mode using a 50 ml columnar reactor which was placed in the oven of a “Spe-ed” SFE unit (Applied Separations, Allentown, US) to control the temperature as illustrated in Figure 1. Experiments were conducted between a temperature range of 160 °C to 220 °C with residence times of 5 to 60 min at pressure of 20 MPa. A mass fraction of 1% of rice bran was used as the feed in these experiments. The acidification by carbon dioxide was also studied as an alternative means to enhance the degree of hydrolysis, CO₂ was pumped in to system by using the system supplied by TharSFC, USA.

3.2 Batch hydrolysis of Sunflower oil, rice bran oil and beef tallow

Batch sub-critical water hydrolysis of vegetable oils and beef tallow was conducted using a 200 ml reactor made of Teflon which in turn was placed in the well of autoclave which was kept at a constant temperature. The effect of oil: water ratio at 1:4 and 1:2.3 were studied at a fixed temperature of 200 °C and at the vapour pressure generated by the reaction temperature. The samples were analysed initially starting at the time it took to reach 200 °C (~ 30 min) and thereafter at 30 min interval for a total duration of 120 min. The stirrer speed was held at 300 r/min, as to use higher agitation rates produced a vortex, which prevented the thermocouple making contact with the solution phase.

3.3 Continuous flow sub-critical water hydrolysis of sunflower oil

Continuous flow sub-critical water hydrolysis of sunflower oil was carried out using a tubular flow reactor (180 ml) which was placed in a muffle furnace (GallenKamp, UK) to provide temperature control. Gilson Model 305 HPLC pumps (Gilson, USA) were used to pump oil:water mixtures in ratios of 1:4 and 1:1 into the reactor. A water cooler LTD20G (Grant Instruments, UK) was used to lower the temperature of hydrolysed oil and pressure was maintained by a back pressure regulating valve. Process optimisation was carried out according to an experimental design based on RSM using a central composite design. The independent variables considered were temperature (°C), pressure (MPa), oil to water ratio (v/v) and residence time (min) as shown in Table 1. The percentage yield (%) was selected as the dependent variable.

3.4 Compositional analysis of hydrolysed rice bran

Carbohydrate analysis was performed under isocratic conditions using an HPLC unit fitted with a Nucleogel Sugar Na form, 300 mm × 7.8 mm column, a Merck-Hitachi L-7100 pump, a Techlab column oven, and a Agilent 1100 RI-detector. Ultra-pure water was used as the mobile phase. The major saccharide components were resolved using a eluent flow rate of 0.5 ml/min and a column temperature held at 70 °C. A 20 µL sample was injected and analytes identified based on their retention times relative to independent injection of external standards.

3.5 Free fatty acid quantification in the hydrolysed oils

To estimate the efficiency of sub-critical water mediated hydrolysis of sunflower, rice bran oil and tallow, the amount of free fatty acid in the samples was determined using two methods: 1) the total acidity titration official method of the American Oil Chemist Society AOCS (Ca 5a-40), 2) a MicroChem II analyser (SafTest system MP Biomedicals, UK). Free Fatty Acids (FFA) were determined spectrophotometrically by using a chromogen reagent which interacts with the FFA and therefore changes their visible spectrum. This is a primary method calibrated with known amounts of FFA. The Limit of Detection (LOD) was 0.02%.

Prior to conducting the free fatty acid assays the components in the hydrolysed oil were separated. The hydrolysed oil or fat sample was poured into a separating funnel with a small amount (1g) of salt (Na_2SO_4), and the oil/water mixture was extracted with equal volumes of diethyl ether two or three times. The ether phase was then evaporated by rotary vacuum distillation to leave a residue consisting of the hydrolysed fatty acids and any unreacted triacylglycerides. Following the method employed by (Holliday et al., 1997) sodium sulphate was added to the water to salt out the fatty acids and break the emulsion that had formed.

3.6 Experimental Design

The central composite rotatable design was adopted to optimise the reaction conditions to achieve maximum hydrolysis of sunflower triacylglycerides (TAGs) present in the oil. Temperature of the reaction X_1 , ($^{\circ}\text{C}$); pressure of the reaction X_2 , (MPa); residence time, X_3 , (min); water to oil concentration, X_4 , (v/v); were selected as the variables to maximize the response (conversion of TG to FFA in the hydrolysed sunflower oil). The experimental design included 21 experiments of four variables at five levels, plus and minus alpha (axial point), plus and minus 1 (factorial points) and a centre point. Table 1 gives the range of variables employed, the actual set of experiments undertaken (experimental runs 1-21) and the percentage conversion as measured by the FFAs obtained.

A second order polynomial equation was developed to study the effects of variables on hydrolysis yield. Equation 1 indicates the effect of variables in terms of linear, quadratic and cross-product dependence terms.

$$Y = A_0 + \sum_{i=1}^N A_i X_i + \sum_{i=1}^N A_{ii} X_i^2 + \sum_{i=1}^{N-1} \sum_{j=i+1}^N A_{ij} X_i X_j \quad [1]$$

Where Y is the yield (%), X_i the variable, A_0 the constant term, A_i the coefficient of the linear terms, A_{ii} the coefficient of the quadratic terms, A_{ij} the coefficient of the cross-product terms and N the number of variables. The coefficients of the equation were determined by using Design Expert 7.0 software (Stat-Ease, USA). The analysis of variance (ANOVA) and the lack-of-fit for the final predictive equation were also studied. ANOVA was used to test the significance and adequacy of the model. The mean squares are obtained by dividing the sum of each of the two sources of variation, the model and the error variance, by the respective degrees of freedom. The Fisher variance ratio was employed to statistically validate how well the factors describe the variation in the data about the mean. It can be calculated from the ANOVA by dividing the mean square of the model variance by the mean square of the error variance. The adequacy of the model is given by the P-value of the 'lack-of-fit' test. If the P for the lack-of-fit test is significant, then the equation selected for the fitting of the experimental data is rejected. The smaller the value of the P the more significant is the corresponding coefficient. The graphical representations of the above equation in the form of 3D plots were also used to describe the individual and cumulative effect of the test variables on the response factors.

4. Results and Discussion

4.1 Subcritical water hydrolysis of rice bran lignocellulosic material

Carbohydrates such as starches, cellulose, and hemicellulose are naturally occurring polymers. Under hydrothermal conditions, polymers undergo hydrolysis to form oligomers and monomers such as glucose and other monosaccharides (Dietmar, 2006). Rice bran is rich in carbohydrates and lignin. Major carbohydrates in commercial bran are cellulose, hemicellulose, and starch and range from 7.7 to 13.1%, 9.6 to 12.8%, 8.7 to 11.4%, and 5 to 15%, respectively (Saunders, 1985).

1 Cellulose consists of glucose units, linked by β -(1 \rightarrow 4) - glycosidic bonds which support the
2 formation of strong intra and inter molecule hydrogen bonds. Cellulose has a high degree of
3 crystallinity, which makes it insoluble in water, however under subcritical conditions cellulose is
4 rapidly hydrolysed low molecular weight oligomers which as a consequence are soluble.
5 According to the Dinjus(Dinjus and Kruse, 2004) reaction scheme, cellulose decomposes under
6 hydrothermal conditions to produce water-soluble products e.g glucose/fructose, this indicates that
7 hydrolysis is the primary step of cellulose decomposition. Hemicellulose is a heteropolymer
8 composed of various monosaccharides, including xylose, mannose, glucose and
9 galactose(Bobleter, 1994). Due to its heterogeneous structure, it is less resistant to hydrolysis than
10 cellulose. According to Bobleter(Bobleter, 1994) hemicellulose is easily solubilised in water at
11 temperatures above about 180 °C.

12 Continuous flow sub-critical water hydrolysis of rice bran was conducted with an initial mass
13 fraction of 1 % suspended solids, for this dilution, assuming pure water, the reaction kinetics were
14 independent of the solids concentration. For the purpose of kinetic modelling the properties of pure
15 water(Bobleter, 1994) were assumed in line with the work carried out by Roginsliki et
16 al(Rogalinski et al., 2008a) for the hydrolysis of rye straw and rye silage. The total organic carbon
17 of rice bran hydrolysate was measured to determine the percentage hydrolysis between 160 °C -
18 210 °C (Figure 2). The results show the level of hydrolysis increased with temperature and reaction
19 time, this can be apportioned to changes in physio-chemical properties of water associated with
20 density, e.g. the dielectric constant, dissociation and reaction constants, which are known to vary
21 with temperatures(Marshall and Franck, 1981). Rice bran when subjected to 150 °C for 60 min
22 resulted in 5% solubilisation of the initial biomass, this is in-line with the results of Ando et
23 al(Ando et al., 2000). The percentage hydrolysis of rice bran progressively increases with
24 temperature e.g. at 210 °C and 60 min, 35% of rice bran was hydrolysed. The work of
25 Sasaki(Sasaki et al., 2003) shows 35% solubilisation of sugar cane bagasse at 200 °C for 20 min in

1 a semi-batch reaction mode, this shows that hydrolysis is dependent on both time and the type of
2 biomass being hydrolysed.

3 The data shows that as the temperature of water increases so does the rate of biomass hydrolysis.

4 The results indicate that sub-critical water can efficiently hydrolyse rice bran, a lignocellulose
5 material, into a mixture of soluble carbohydrate moieties at elevated temperatures.

6 In addition to sub critical water-mediated hydrolysis of biopolymers, it is known that the
7 hydronium ion can act as a catalyst to accelerate the decomposition of carbohydrate
8 monomers(Heitz et al., 1986). During the hydrolysis of carbohydrate polymers, not only free
9 sugars are formed, but also there are well documented example of non-targeted side products such
10 as furfural, 5-hydroxymethyl furfural (HMF), formic acid, mixed carboxylic acids and phenolic
11 compounds(Kamio et al., 2006).

12 A range of organic acids were analysed for to determine the level of non-specific reactions. Figure
13 3 illustrates the that at 200 °C, 20 MPa and 20 min of reaction time, xylose degrades into a range
14 of side products. A 25% yield of xylose was obtained after 40 min, its yield decreasing with time
15 as it started to break down into furfural which was then further degraded to formic acid with
16 extended residence time. The results are comparable to the studies of Rogalinski(Rogalinski et al.,
17 2008b) who demonstrated that up to 10% of furfural was generated under SCW mediated
18 hydrolysis of rye straw at 120 °C in 10 min.

19 A detailed knowledge of product information is important to optimise reaction conditions with
20 respect to selective production of desired compounds. It can be inferred that xylose, being the
21 monomeric building block, could be detected in significant amounts. In addition,
22 hydroxymethylfurfural (HMF) was formed as a secondary reaction product during rice bran
23 hydrolysis. Under the parameters study xylose and furfural are major reaction products of rice
24 bran hydrolysis. These compounds were, however not stable under operating conditions due to
25 secondary decomposition reactions.

1 **4.2 Catalytic influence of CO₂ addition**

2 In order to study the impact of carbon dioxide addition on the efficacy of sub-critical water
3 mediated hydrolysis of rice bran, gaseous CO₂ was added to the reaction medium to generate
4 carbonic acid which should accelerate catalytic cleavage of the carbohydrate polymers. The
5 influence of acidification by carbon dioxide as a function of temperature is depicted in Figure 2
6 and compared to rice bran hydrolysis in pure water. The addition of carbon dioxide appears to
7 support an enhanced rate of hydrolysis at 150 °C and 180 °C, whereas at 210 °C the same catalytic
8 effect is not observed. A similar trend was also shown by Rogalinski(Rogalinski et al., 2008b)
9 using pure cellulose as a substrate where the addition of CO₂ results in an enhancement rate of
10 cellulose hydrolysis at 240 °C, but this catalytic effect diminishes at 260 °C and 280 °C. (In the
11 case of rice bran a complex lignocellulose material the effect appears to starts at lower temperature
12 i.e. 210 °C) . The decrease in catalytic influence of carbon dioxide with increasing temperature
13 could be attributed to the following: Firstly - the associated drop in pH of saturated water/CO₂
14 mixtures decreases with increasing temperature, leading to a less pronounced rate of enhancement
15 compared to the already increased rate of reaction at higher temperatures. Secondly - acidic
16 compounds are rapidly formed in the course of reaction e.g. organic acids, therefore the low pH
17 values cannot be attributed directly to carbonic acid in summary the dissociation of carbonic acid
18 under the operating conditions has a reduced impact on the pH compared to the acidic compounds
19 formed during the reaction. Auto acidification, which is a function of the type of biomass, could
20 account for the verifiability of the impact of CO₂ on the efficacy of sub-critical water mediated
21 hydrolysis found within the literature(Peter van Walsum and Shi, 2004).

22

23 **4.3 Kinetic modelling of carbohydrate hydrolysis**

24 From Figure 2 it can be concluded that the data can be correlated by a linear regression fit, which
25 suggest the reaction kinetics is first order. The experimental data was further modelled using

global rate law which again confirmed that first order reaction kinetics could be applied. This modelling approach is especially applicable at lower temperatures, where the data obey a linear relationship. The reaction rate constants at different temperatures can be derived by determining the values of the slope of the straight lines (values not shown). By adopting the Arrhenius law for the reaction kinetics the pre-exponential factor (K_0) and activation energy (E^a) of the reaction can be determined, $\ln[k(T)]$ versus the reciprocal temperature and reading the ordinate intercept and the slope of the straight line (Figure 4).

The straight line was obtained by linear regression of the reaction rate constants at different temperatures. As can be seen, the calculated straight line is in accordance with the value of the reaction rate constants. Hence, the Arrhenius' law can be applied to the liquefaction of rice bran in sub critical water. Rogalinski(Rogalinski et al., 2008b) also described satisfactorily, the hydrolysis kinetics of biopolymers in sub critical water by a global first order rate law.

The kinetic modelling for the hydrolysis of rice bran in the sub-critical water/ CO_2 system is based on pure water, and no carbon dioxide concentration dependence was assumed. As indicated in Figure 4, the reaction rate constants for rice bran hydrolysis in the presence of CO_2 are higher than those for pure water at temperatures of 150 °C to 210 °C. The lines in Figure 4 corresponding to Arrhenius based-kinetics approach one another with increasing temperature, so that no further enhancement in the rate of liquefaction could be obtained by adding carbon dioxide at temperatures higher than 220 °C. The addition of carbon dioxide however into the SCW increases the liquefaction yield at the same temperatures.

In summary from empirical results combined with kinetic modelling it can be concluded that the kinetics of lignocellulosic conversion in sub-critical water can accurately be described by a global first order rate law and corroborates the work of Rogalinski(Rogalinski et al., 2008b).

4.4 Batch hydrolysis of lipid-containing substrates

Batch hydrolysis of sunflower oil, rice bran oil and beef tallow were conducted at 200 °C at vapour pressure. All three oils had different initial free fatty acid (FFA) content - sunflower oil (0.81%), rice bran oil (12.09%) and beef tallow (17.60%) before commencing SCW hydrolysis, largely due to poor storage conditions or thermal abuse. The effect of oil: water ratios and pressure were studied for beef tallow. From Figure 5a it is clear that the degree of lipid hydrolysis and therefore fatty acid release increased with extended residency time. However the results of the sub-critical water mediated hydrolysis indicates that the three oils have different induction periods. Sunflower oil, which is a refined oil and therefore has very low levels of FFA, exhibits longest induction period of the three oils tested. The consequence of the extended induction period during the hydrolysis of the sunflower oil is that the total yield of FFA after 140 min is 30%, compared to approximately 70% for the other oils. Induction periods during hydrolysis of oils has been observed during industrial fat splitting processes. Lascaray(Lascaray, 1952) provides a detail explanation of the phenomena. In summary, the induction period has been attributed to the combination of two factors 1) the initial level of free fatty acids which have the potential to auto-catalyse the reaction and 2) the solubility of water in oil which is a function of temperature. From the hydrolysis data, it is clear that the induction period observed with sunflower oil, can be accounted for by explanations offered by Lascaray(Lascaray, 1952) as the oil has the lowest level initially of free fatty acids. At the start of hydrolysis the reaction is thought to be heterogeneous and occurs at the oil water interface, as the level of free fatty acids increase the reaction becomes homogenous thereby promoting the maximal rate of hydrolysis. According to Lascaray and Mills (Lascaray, 1952; Mills and McClain, 1949) when the level of free fatty acids in the reaction medium represent 15-20% there is very little induction period, this is reflected in the results with the tallow which has 21% free fatty acid. Therefore hydrolysis of lipid is a complex interplay between the availability of acid catalysts and water oil solubility.

4.5 Optimization of reaction conditions for continuous sunflower oil hydrolysis using response surface methodology

In the present study, optimization of conditions for hydrolysis of sunflower oil was carried out using the CCRD (Central Composite Rotatable Design). Table 1 lists the independent variables, the experimental design and, observed and predicted responses. The estimated response model equations were obtained as shown below (temperature X1, °C; pressure X2, MPa; residence time X3, min; water to oil concentration X4, v/v)

$$\text{Yield(\%)} = 376.3147 - 2.7309 \times X1 - 1.0898 \times X2 - 0.4232 \times X3 + 2.0049 \times X4 + 0.0039 \times X1 \times X2 + 0.0020 \times X1 \times X3 - 0.0029 \times X1 \times X4 + 0.0007 \times X2 \times X3 - 0.0025 \times X2 \times X4 + 0.0023 \times X3 \times X4 + 0.0043 \times X1^2 + 0.0006 \times X2^2 - 0.0031 \times X3^2 - 0.0044 \times X4^2 \quad [2]$$

The results of using ANOVA for fitting the second order response surface model by the least squares method are presented in Table 2. The high F value (F model = 292.18) with a very low probability value (P < 0.0001) indicates the model has a significant fit. The significance of all coefficients was established by P-values shown in Table 2. The smaller the P value for a parameter the more significant is impact of the experimental parameter (Khuri and Cornell, 1987). The linear terms; temperature, pressure and residence time, and the quadratic terms; squared reaction temperature (P-value = <0.0001) and residence time (P-value = 0.0938) made the most significant contributions to the fitted model. Furthermore, all first order interaction effects of the four predictors were statistically significant, with the greatest significance being the interaction of temperature × pressure (P-value = <0.0001) and temperature × residence time (P-value = 0.0015). At the same time, the low value of the coefficient of variation (CV = 6.19%) indicates that results of the fitted model are reliable. A high value of $R^2 = 0.9985$ is an indication of high precision of

the model fitted. The value of the adjusted coefficient of determination (R^2 Adj = 0.9951) also indicates an excellent correlation, supporting that this model explains the experimental results adequately.

Equation 2 was optimised to identify the optimum process conditions for maximizing the FFA yield. The process conditions predicted to yield nearly 100% FFA were: temperature 385 °C; pressure 20 MPa pressure; residence time of 35 min; oil to water ratio 1:1.8 (v/v) represents 65% water. Experiments conducted under these conditions yielded 93.5%, FFA which is in correlation with the predicted results shown in Table 1.

Three dimensional surface plots graphically represent the relationship between the responses and process parameters, and are presented in Figures 8a-c. The plots were made by taking an infinite number of combinations of the values of the two test variables at a time and keeping the values of the remaining two test variables constant. Such plots are useful for understanding the interaction of two test variables and determining their optimum levels by holding the other test variables constant.

4.6 The effect of temperature, pressure, residence time and oil to water ratio on lipid hydrolysis

The trends noted in Figure 5b can be explained by the fact that the vegetable oil is insoluble in water at temperatures below 250 °C (Holliday et al., 1997), hence the reaction under these conditions is extremely slow. Increasing the temperature promotes the solubility of oil in water. hence the rate of hydrolysis increases (Lawrence, 1954). As noted in Figure 5b, at 270 °C, the hydrolysis does not start until 20 min has elapsed and as consequence it took 80 min to reach 50% hydrolysis. This effect cannot be seen in the graphical representation due to the noise level in the model, whereas it can be clearly seen in table 1. In, comparison at the higher temperatures; 290 °C, 310 °C and 330 °C the time taken to reach 50% hydrolysis was 20 min. At 290 °C the initial rate

was higher than 270 °C, still after 90 min, a plateau was reached, however for the 310 °C and 330 °C the approximately 90% conversion was achieved after 45 min and 25 min, respectively.

The results clearly indicate that the optimal temperature required for effective hydrolysis of lipid was 385 °C. It was observed that, if the temperature was increased above the optimum level, there was a decrease in FFA yield, indicating perhaps a thermal transformation of FFA or the esterification of fatty acids with glycerol(Holliday et al., 1997).

The effect of higher reaction temperature on hydrolysis of lipids has been discussed in detail by several investigators (Pinto and Lanças, 2006; Sturzenegger and Sturm, 1951) at temperatures up to 280 °C. As with our results Holliday(Holliday et al., 1997) found the degree of hydrolysis increases with increase of temperature, however, the washing of the glycerol from the oil phase tends to become more and more of a rate controlling factor thus limiting the advantages to be obtained from further increases of temperature. The observed hydrolysis in the above range of temperatures has not been reported previously except by Holliday(Holliday et al., 1997) who performed the experiments in batch mode, this is why this study looked at an increased range of temperatures.

In order to determine the effect of pressure, experiments were conducted at 10 MPa and 20 MPa while keeping the temperature constant at 310 °C and the initial reactant ratio at 1:1 for oil and water. RSM models incorporating this data are shown in Figures 6a-c indicate that at a pressure of 20 MPa with other variables at their optimum levels shows the maximum yield was obtained. The batch hydrolysis of beef tallow (Figure 5a) also indicated a similar effect of pressure. Therefore, our results provide corroborating data suggesting that pressure has a direct influence on the interaction between oil and water.

In Figure 6a the influence of pressure and reaction temperature on sunflower oil hydrolysis is shown. At low temperatures the lipid hydrolysis increased with pressure. Minami(Minami and

1 Saka, 2006) have shown sub-critical water mediated hydrolysis of rapeseed oil reaches a maximum
2 conversion of 90 % at a temperature of 270 °C and 20 MPa for 60 min.

3 Figure 6b shows the effect of pressure and residence time on the conversion of lipid to FFA. Here
4 again, an increase in pressure caused an increase in FFA yield. The reaction time also exerted a
5 significant effect on FFA yield. The model suggests that during the first 20 min of the reaction the
6 rate of hydrolysis was 0.8 percent per second of the change in FFA concentration, thereafter it
7 increased at 1.6 percent per second. The study of Ackelsberg(Ackelsberg, 1958) shows a
8 temperature increase of 10 °C increases the rate of reaction by a factor of 1.2 to 1.5, similar to rate
9 increases as a function of temperature reported by other authors(Pinto and Lanças, 2006;
10 Sturzenegger and Sturm, 1951). Figures 5a and 5b clearly show an increase in FFA conversion as a
11 result of the extended reaction time. The continuous flow process has advantages over the batch
12 process as it gives a higher yield under similar process conditions.

13 The effect of oil to water ratio for the batch hydrolysis of beef tallow is depicted in Figure 5a. With
14 an oil/water ratio of 1:4 the percentage conversion of lipid to FFA was 75 % which decreased by
15 10% when the ratio was reduced to 1:2.3. Continuous flow sub-critical water mediated hydrolysis
16 gave similar trends which are shown in Figure 6c. Even at a lowest pressure of 10 MPa, 30% FFA
17 yield was observed with an oil/water ratio of 1:4. The oil/water ratio effect is positive and this is
18 the limiting factor for the reaction. An excess of water is essential to wash out of the oil phase
19 glycerol as it is produced, allowing the reaction to go to completion as shown by Mills(Mills and
20 McClain, 1949). There are however, other factors to be considered that impose an upper limit on
21 the oil to water. The ratio normally employed is between 70% and 90% corresponding to about 14
22 times the stoichiometric amount of water.

23 The position of equilibrium, in accordance with the law of mass action, will depend on the
24 concentration of glycerol in the oil phase. Glycerol, which is practically insoluble in the dry fats,
25 becomes soluble to some extent when fats contain dissolved water. From the data

published(Lascaray, 1952) it can be seen that a fat in equilibrium with a water glycerol solution dissolves glycerol and water in such a proportion that the glycerol/water ratio is same in both the phases. Thus a lipid with 10% dissolved water in contact with a solution of 15% glycerol will contain 1.5% free glycerol in solution. The negative influence of glycerol on the progress of the hydrolysis reaction is more pronounced when the reaction approaches equilibrium. As lipid is converted to fatty acid, the concentration of the reacting ester decreases and the reaction slows down. The amount of free glycerol is at this stage increasing and tends to suppress further reaction(Lawrence, 1954). Since glycerol is water-soluble, the reaction can be driven towards completion if the glycerine is removed from the oil into the water phase as quickly as possible and replaced by more water.

5. Conclusion

Sub-critical water is widely accepted as an environmentally benign solvent, for extraction but also as a catalytic medium therefore has the potential to support processing of multiple components found in biomass. The present work focused on the optimisation of key process parameters for the hydrolysis of both lignocellulosic material and a range of lipids. Results from both batch and continuous processing show carbohydrate polymers and triacylglycerols can be efficiently (> 90%) hydrolysed within 60 min. Reaction kinetics and RSM models were created and successfully validated, thereby confirming the utility of the RSM as a tool to assist in process development.

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Figure1
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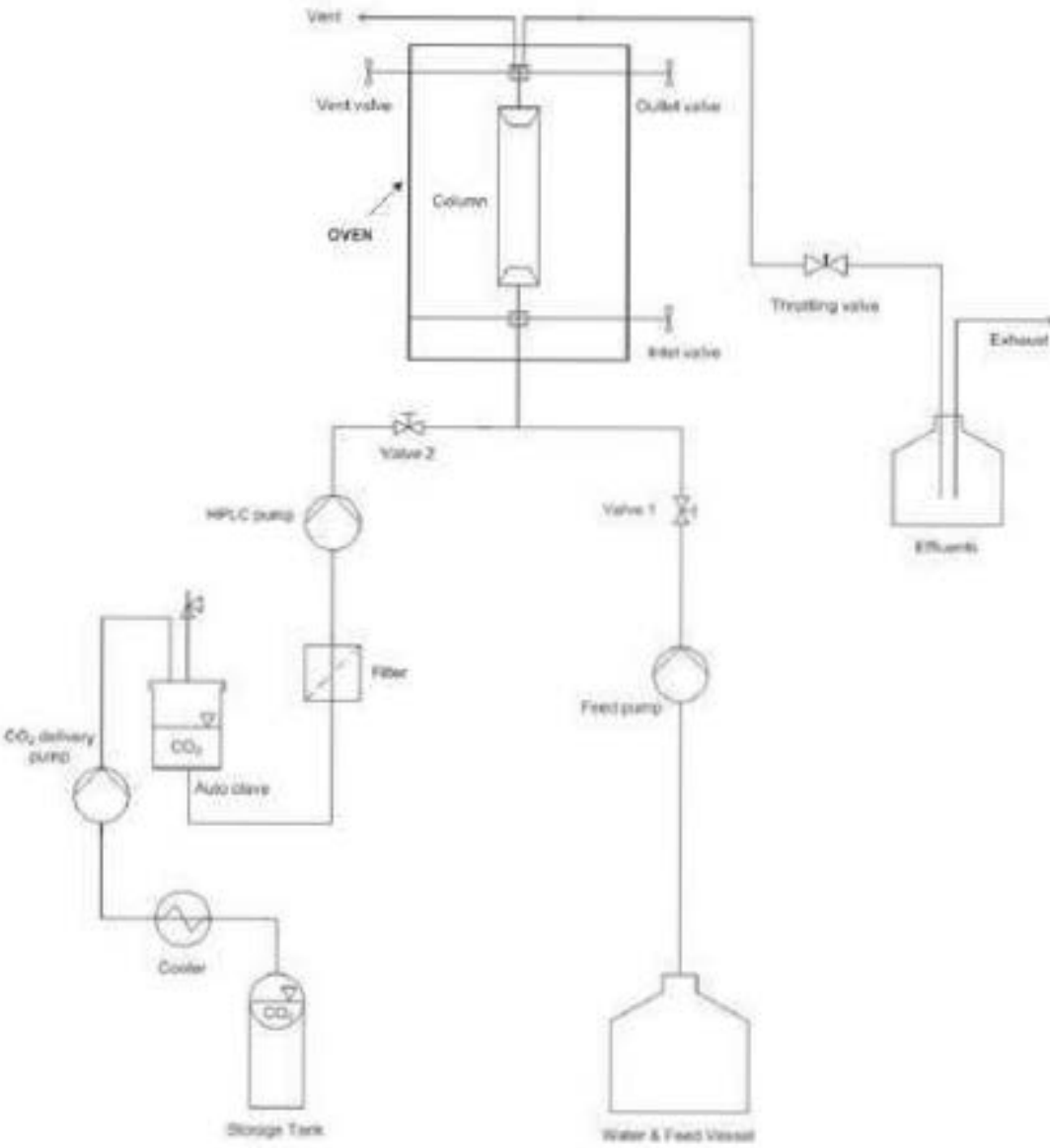


Fig. 1- Schematic diagram for continuous flow hydrolysis with CO₂ acidification option.

Figure2
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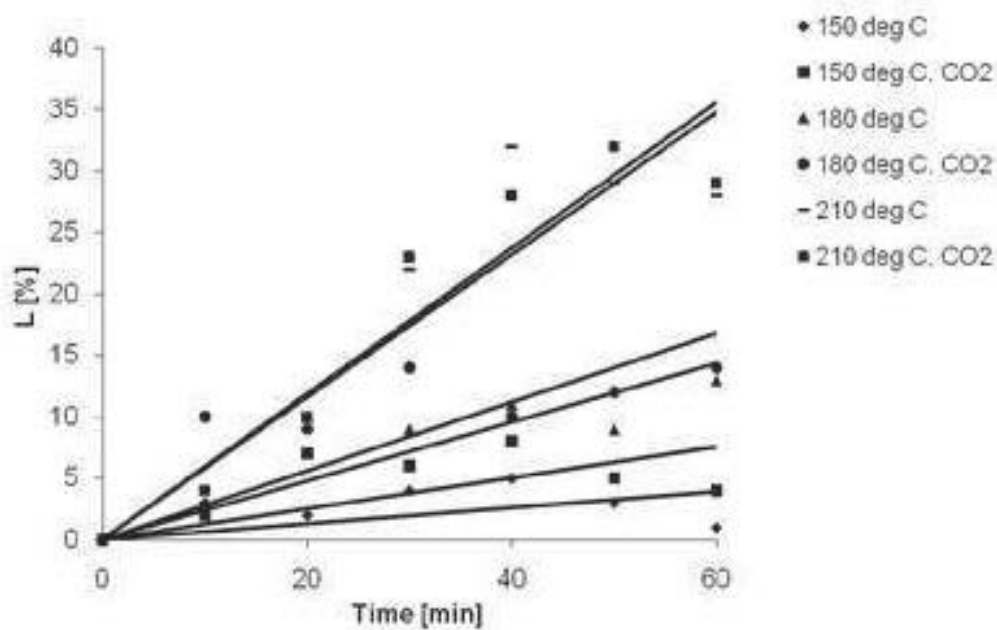


Fig. 2- Effect of temperature and CO₂ on the percentage hydrolysis of rice bran in a piston reactor at a pressure 20 MPa

Figure3
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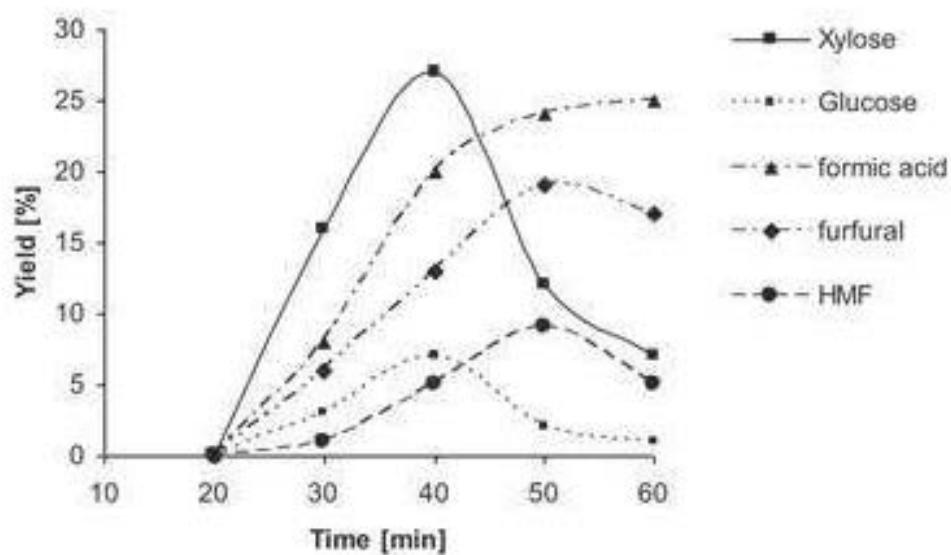


Fig. 3- Percentage yield of side products after 20 minutes in function of time at 20 MPa @ 200 °C for the hydrolysis of rice bran with SCW.

Figure4
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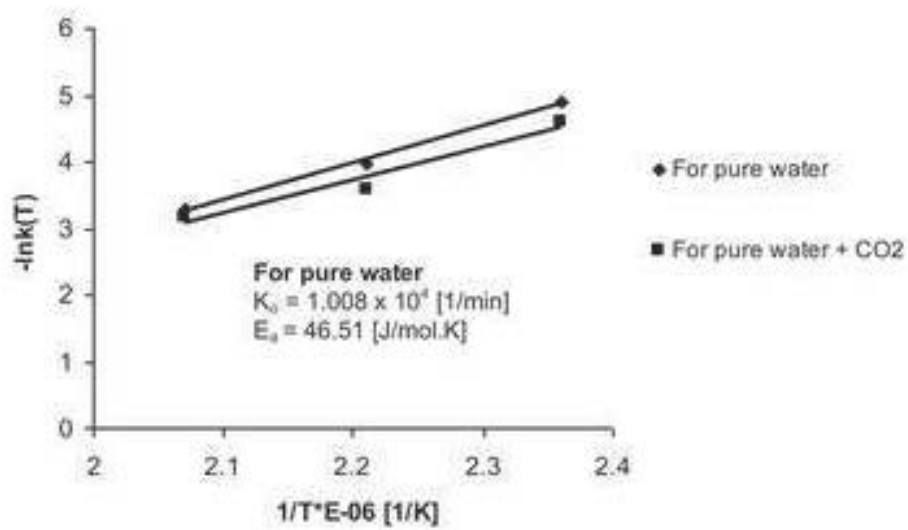


Fig. 4- Arrhenius plot for water and water/carbon dioxide mixtures

Figure5
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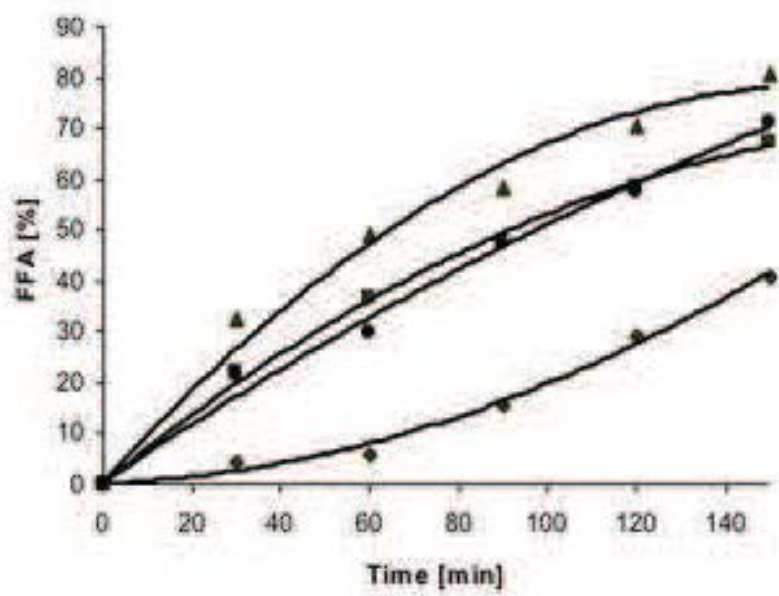


Fig. 5a. Percentage conversion of triglyceride to FFA with residence time during batch hydrolysis of sunflower oil (SO), rice bran oil (RBO) and beef tallow (BT) @ 200° C at ambient pressure with oil/water ratio of 1:4 ♦ A) SO, • B) RBO, C) • BT, • D) BT with oil/water ratio of 1:2.3

Figure6
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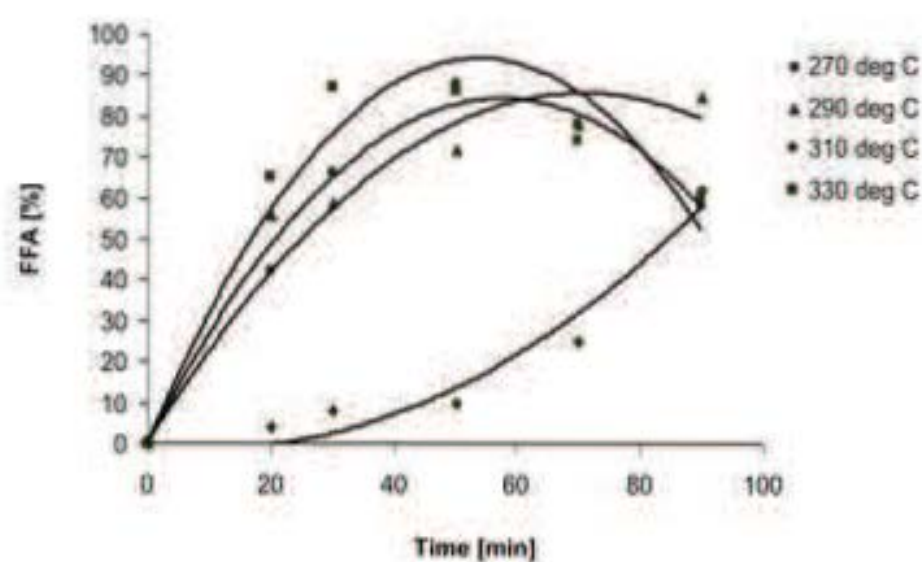


Fig. 5b- Percentage conversion of triglyceride to FFA in continuous flow hydrolysis process with oil to water ratio of 1:4 at 20 MPa

Figure7
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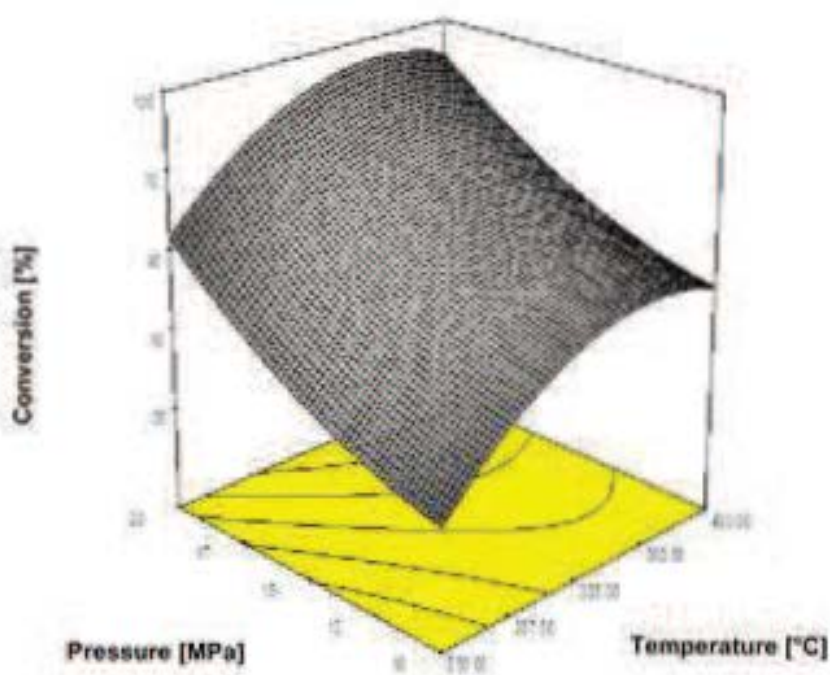


Fig. 6a- Plot shows the mutual effect of temperature and pressure at residence time of 35 min and oil to water ratio of 1:1.8 v/v on percentage conversion of TG to FFA

Figure8
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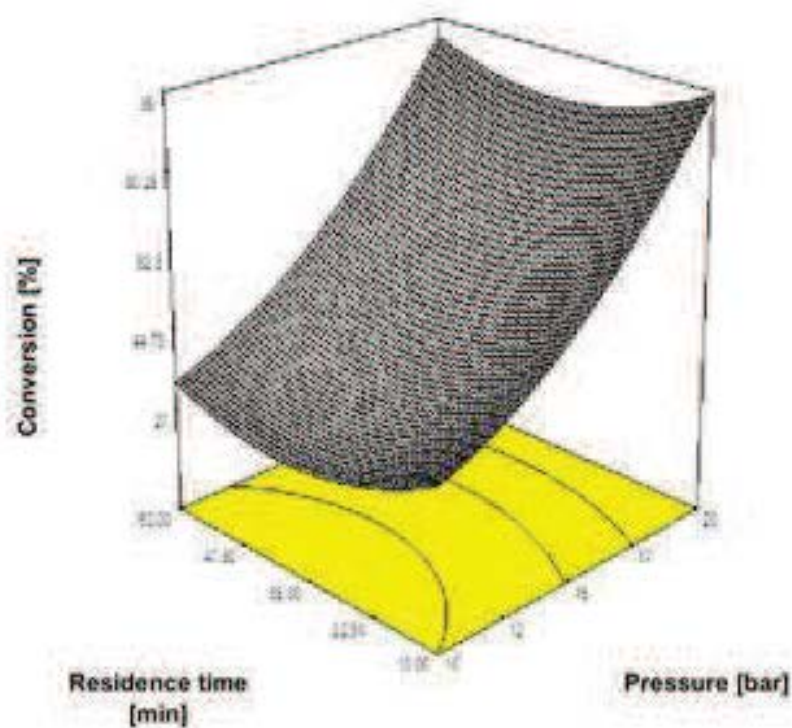


Fig. 6b- Plot shows the mutual effect of pressure and residence time at 330 °C and 1:1.8 v/v oil to water ratio on percentage conversion of TG to FFA.

Figure9
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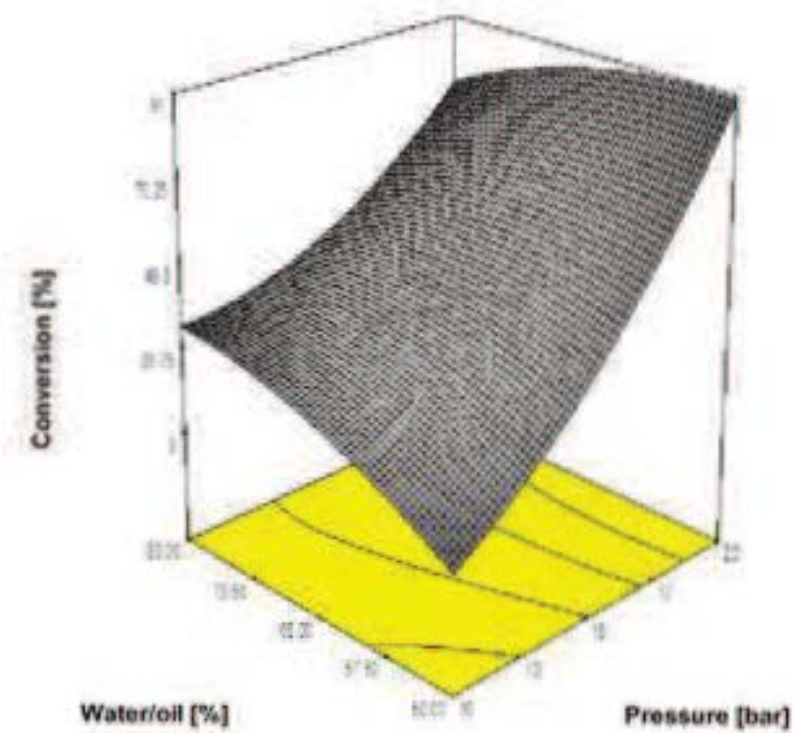


Fig. 6c- Plot shows the mutual effect of pressure and oil/water ratio-in percentage at 330 °C and residence time of 35 min on percentage conversion of TG to FFA

Table1

Run No.	Process Variables				Yield (%)		Relative deviation (%)
	X1	X2	X3	X4	Predicted	Experimental	
1	250	20	60	80	14.49	15.28	2.67
2	310	15	35	65	19.05	18.33	1.93
3	250	10	10	50	1.48	1.65	5.54
4	250	20	10	80	4.44	4.00	5.23
5	250	10	60	50	4.52	5.41	8.95
6	310	15	35	50	14.58	15.28	2.34
7	370	20	60	50	89.47	90.71	0.69
8	310	15	60	65	23.46	22.80	1.42
9	310	15	10	65	10.66	13.40	11.38
10	370	10	60	80	49.42	50.29	0.87
11	370	15	35	65	59.53	60.63	0.92
12	310	15	35	65	19.05	18.80	0.67
13	370	20	10	50	70.43	70.03	0.28
14	310	15	35	65	19.05	18.33	1.93
15	250	15	35	65	9.59	10.11	2.66
16	370	10	10	80	30.38	30.55	0.29
17	310	20	35	65	29.56	30.32	1.27
18	310	15	35	80	21.54	22.56	2.32
19	310	15	35	65	19.05	20.92	4.67
20	310	10	35	65	11.56	12.93	5.60
21	310	15	35	65	19.05	20.45	3.54
22 ^a	385	20	35	65	99.85	93.53	3.27

^a Validation experiment at optimal conditions

Table 1- Experimental design showing actual variables studied along with the experimental and predicted yield (temperature X1, °C; pressure X2, MPa; residence time X3, min; water to oil concentration X4, v/v)

Table2

Source of variation	Sum of Squares	Degrees of freedom (df)	Mean Squares	F Value	p-value Prob >F
Model	10489.19	14	749.23	292.18	< 0.0001
X1-Temperature	1250.00	1	1250.00	487.47	< 0.0001
X2-Pressure	162.00	1	162.00	63.18	0.0002
X3-Res. Time	409.60	1	409.60	159.73	< 0.0001
X4-water/oil ratio	24.50	1	24.50	9.55	0.0214
X1.X2	225.63	1	225.63	87.99	< 0.0001
X1.X3	78.13	1	78.13	30.47	0.0015
X1.X4	11.03	1	11.03	4.30	0.0835
X2.X3	6.13	1	6.13	2.39	0.1732
X2.X4	5.63	1	5.63	2.19	0.1891
X3.X4	6.13	1	6.13	2.39	0.1732
X1 ²	613.83	1	613.83	239.38	< 0.0001
X2 ²	5.79	1	5.79	2.26	0.1835
X3 ²	10.15	1	10.15	3.96	0.0938
X4 ²	2.52	1	2.52	0.98	0.3598
Residual	15.39	6	2.56		
Lack of Fit	10.59	2	5.29	4.41	0.0973
Pure Error	4.80	4	1.20		
CV= 6.19%	R ² =0.9985	R ² _{Adj} =0.9951	Predicted R ² =0.7718		

Table 2- Analysis of variance ANOVA for response surface quadratic model



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journal homepage: www.elsevier.com/locate/resconrecStudy on sub-critical water mediated hydrolysis of *Miscanthus* a lignocellulosic biomassR.M.N. Roque^{a,*}, M.N. Baig^a, G.A. Leeke^a, S. Bowra^b, R.C.D. Santos^a^a School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK^b Phytatec (UK) Ltd., Plas Gogerddan, Aberystwyth SY23 3EB, UK

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ABSTRACT

The present study was undertaken to evaluate the impact of sub-critical water (Sub-CW) processing parameters, such as temperature and biomass:Sub-CW:ethanol:CO₂ ratio, in order to optimise the delignification of the *Miscanthus* χ giganteus, a lignocellulosic biomass.

The percentage lignin solubilised (delignification) was calculated by measuring the amount of lignin, using the Klason assay, that remained in the insoluble fraction. The amount of biomass solubilised was determined by calculating the difference between the initial weight of biomass and the weight of the residual insoluble fraction.

Experimental results showed a maximum solubilisation and delignification of 53% and 86% respectively at 200 °C and biomass/solvent ratio of 1:100, i.e., 2.5 g in 250 ml of water:ethanol mixture (50:50).

Scanning Electron Microscopy (SEM) images were taken to analyse the effect of hydrolysis on cellulose fibres structure. The results showed the residual fibres appeared intact, with some lignin globules attached to them. Consequently, it was concluded that the use of Sub-CW:ethanol:CO₂ mediated hydrolysis under the stated operating conditions supported delignification without destroying the cellulose fibres.

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1. Introduction

Forestry, short rotation crops, e.g., willow and specific energy crops, e.g., *Miscanthus* and Switch grass are being developed to meet the solid fuel/co-firing requirements for existing power generation. The energy crops are a source of biomass dominated by three naturally occurring polymers, hemicellulose, cellulose and lignin, which are found in close association and make up the cell walls. The three polymers are collectively referred to lignocellulose. All plant biomass contains a proportion of lignocellulose, but crop residues (husks) and specific energy crops have a higher proportion of these natural polymers (Murphy and McCarthy, 2005). The current first generation of bioethanol production is driven by starch fermentation. However the cellulose 'locked' up in lignocellulosic biomass represents an abundant and cheaper source of carbohydrate that could be used to produce bioethanol and at the same time increase overall economic viability.

As a first step, there is a need to develop environmentally benign yet efficient that can hydrolyse lignocellulose biomass to enable separation and recovery of the principle component, i.e., cellulose, hemicellulose and lignin. Furthermore, lignin, a fermentation by

product, is the most abundant source of aromatic compounds in nature (Toledano et al., 2010) therefore the efficient separation and purification of lignin could potentially further enhance the economics of bioethanol production (Toledano et al., 2010). Lignin has traditionally many applications and is currently sourced from the paper pulp industry as lignosulphates. However, if lignin could be sourced free of sulphate, the macromolecule could find new innovative uses in polymeric materials, resins, composites, and also generate a large range of chemical intermediates to replace those derived from oil (Toledano et al., 2010).

Sub-CW holds significant promise as a 'green' alternative to the use of corrosive acids and organic solvents, which are used industrially at present (Hashaikeh et al., 2007). Sreenath et al. (1999) has shown that at higher temperatures, e.g., 220 °C, Sub-CW can dissolve hemicelluloses completely and remove lignin partially within 2 min with no chemicals used. Although, to increase the efficiency of Sub-CW mediated hydrolysis of biomass, pressurised carbon dioxide has been used to acidify the medium through the formation of carbonic acid (van Walsum et al., 2007; van Walsum and Shi, 2004). At the end of the hydrolysis the carbonic acid can be converted again to carbon dioxide by releasing the pressure in the reactor allowing carbonic acid to sublime. Carbonic acid is not a strong acid comparatively to sulphuric acid used in acid mediated hydrolysis and hence does not offer the same hydrolytic capability of sulphuric acid (van Walsum et al., 2007; van Walsum and Shi,

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2004). However, van Walsum et al. (2007) have demonstrated that at temperatures in the order of 200 °C, carbonic acid does exhibit a catalytic effect on the hydrolysis of xylan.

In conclusion, Sub-CW mediated hydrolysis of lignocellulosic biomass, reduces the need for chemicals the neutralisation of the resulting hydrolysate. Thus lower amounts of neutralisation residues compared to many processes such as dilute-acid pre-treatment. In the dilute-acid pre-treatment usually $\text{Ca}(\text{OH})_2$, NaOH or KOH are used to neutralise the hydrolysate medium producing precipitates of sulphate salts as residues. In addition, the use of NaOH and KOH was mentioned to cause growth inhibition due to osmotic effects during the fermentation steps for bioethanol production (Neureiter et al., 2004; Taherzadeh and Karimi, 2008). In addition to that, Sub-CW mediated hydrolysis is also seen as a path to optimise energy usage, since it does not require extra energy for a subsequent water evaporation step in comparison to systems based on biomass where the moisture content in biomass is the main limitation. This is the case when using direct combustion and thermal gasification, where a large amount of energy is consumed in order to evaporate the water content (Hashaikh et al., 2007).

Previous studies, have evaluated the effect of pressure, temperature and the solvent composition on lignocellulosic biomass hydrolysis and delignification. Li and Kiran (1988) reported the treatment of red spruce wood with a ternary system of carbon dioxide:water:ethanol (mole fractions of CO_2 and H_2O of .91 and .022 respectively) at 190 °C and 290 bar the percentage of the wood solubilised was 19.3% under these conditions. Pasquini et al. (2005) applied similar conditions to optimise delignification of sugar cane bagasse and *Pinus taeda* wood chips. The best results were obtained at 16.0 MPa and 190 °C with a delignification extent of 93.1% for *P. taeda* wood chips and 88.4% for sugar cane bagasse. In addition, temperature proved to have a much greater influence than pressure, although the catalysing effect of CO_2 in starch and cellulose hydrolysis is referred by Rogalinski et al. (2008) to be reduced with the increasing of temperature.

The objective of our work was to study the effect of ethanol and carbon dioxide as modifiers on the efficacy of Sub-CW mediated hydrolysis of the lignocellulosic biomass but more specifically delignification of the biomass, i.e., the extraction and solubilisation of lignin such that, in future lignin can be recovered from the liquid fraction after hydrolysis.

2. Materials and methods

2.1. Sample

Air-dried *Miscanthus x giganteus* was kindly provided by Phytatec (UK) Ltd. Ethanol absolute (Fisher Scientific, UK) and carbon dioxide (vapour withdrawal, BOC, UK) used had both analytical grade.

2.2. Sample preparation

Prior to hydrolysis the samples (2.5, 5, 10, 15 g) were imbibed in 250 ml mixture of water:ethanol (50:50) at 50 °C for 20 min before grinding in a blender (Moulinex Vitamix Y42, 400 W, 1.5 l) for 3 min. The grinding conditions had previously been optimised by determining the best temperature, soaking time, grinding time and solid:liquid ratio and it was shown that the average particle size was 500 μm after filtration and drying of the slurry produced during the grinding tests.

2.3. Biomass hydrolysis

The pH of the ground biomass mixture in the water:ethanol solvent, was measured before transferring the mixture to a stirred high-pressure vessel (500 ml high-pressure stirred reactor manufactured in Alloy C276 by Parr). The reactor was then closed and pressurised to 55 bar (cylinder pressure) with carbon dioxide. After that, temperature was increased to the desired temperature where it was kept stable during the reaction time (1 h) by checking it with the reactor 4836 Parr controller. To stop the reaction the high-pressure vessel was quenched both by removing the heating jacket and by opening the cooling system, which consists of a cooling coil inside the pressure vessel where a coolant flows at -7°C . Then the vessel was depressurised and opened. Final pH was measured before the resulting insoluble fraction was recovered by vacuum filtration through a sintered disc, porosity 1, rinsed with mixture of water:ethanol (50:50) and dried at 105 °C.

2.4. Klason lignin determination

After the hydrolysis experiments, the residual insoluble fraction was analysed for lignin content using the Klason assay following the ASTM Standard method E 1721-01 (Determination of Acid-insoluble Residue in Biomass) and the amount of Klason lignin remaining in the insoluble fraction was compared to the amount present in the biomass giving the percentage lignin solubilised.

2.5. SEM images

SEM images of *Miscanthus* fibres before and after the hydrolysis treatment were performed at the Centre for Electron Microscopy, University of Birmingham using a Philips XL-30 FEG ESEM scanning electron microscope operating at 10 kV. Prior to imaging, samples were coated with gold for 120 s using a gold sputter coater, Polaron SC 7640.

3. Results

3.1. Efficacy of Sub-CW:ethanol: CO_2 mediated hydrolysis on biomass solubility

Experiments were conducted to evaluate the impact of temperature and biomass:solvent ratio on the efficacy on Sub-CW:ethanol: CO_2 mediated hydrolysis of *Miscanthus*. To determine the degree of biomass hydrolysis, the weight of the soluble fraction was estimated by subtracting the weight of residual insoluble fraction from initial weight of biomass the results were then expressed as percentage of the initial weight of biomass (Fig. 1).

The results in Fig. 1 show biomass solubility varies in a range between 35 and 53%.

The temperature effect on biomass solubility was determined to a significant $P=0.0001$ with a confidence level of 95%, while the effect of biomass:solvent ratio was not significant $P=0.78$.

3.2. Efficacy of Sub-CW:ethanol: CO_2 mediated hydrolysis on biomass delignification

The insoluble fraction resulting from the biomass hydrolysis was analysed by Klason lignin and the results were compared to the amount of Klason lignin present in the raw material, which is 26%.

The percentage of biomass delignification (Fig. 2) was in the range 58–86%.

The effect of temperature on biomass delignification was significant $P=0.003$. Therefore, it is possible to consider if the biomass:solvent ratio is fixed the biomass delignification increases on average 18% when the temperature changes from 180 to 200 °C.

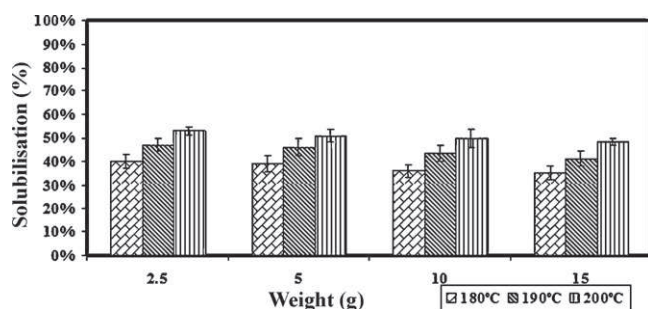


Fig. 1. Degree of biomass solubilisation at different hydrolysis conditions. Results were based on the weight of the soluble fraction expressed as percentage of the initial weight of biomass.

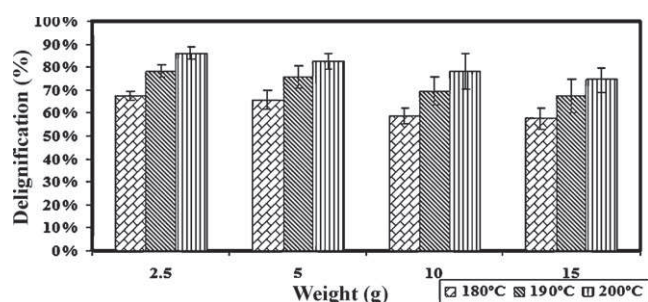


Fig. 2. Degree of biomass delignification at different hydrolysis conditions. Results were determined per each experiment, comparing the initial amount of Klason lignin in biomass to the amount of Klason lignin present in the insoluble fraction.

The effect of biomass:solvent ratio was not significant $P=0.44$.

While the effect of biomass:solvent ratio did not have a significant impact on biomass delignification, there was a slight tendency for percentage delignification to be reduced when the weight of biomass was increased.

Table 1

pH values of the reaction medium before and after hydrolysis at different conditions.

Weight (g)	180 °C		190 °C		200 °C	
	pH _{initial}	pH _{final}	pH _{initial}	pH _{final}	pH _{initial}	pH _{final}
2.5	6.6	4.8	6.0	4.5	6.2	4.3
5	6.2	4.6	6.1	4.3	6.1	4.3
10	6.3	4.7	6.3	4.6	6.4	4.3
15	6.2	4.7	6.2	4.5	6.1	4.2

3.3. pH of the medium

The pH value of the reaction medium was measured before and after each hydrolysis reaction. Results are shown in Table 1.

Table 1 shows a low variability of the pH values among the different conditions. The difference between initial and final pH values of around 2 pH units can be attributed to either the CO₂ added during the reaction, which produces carbonic acid or the production of organic acids during the hydrolysis reaction.

3.4. SEM images – qualitative fibre analysis

The quality of the cellulose fibres left in the insoluble fraction after hydrolysis at 200 °C and biomass load of 15 g was evaluated by Scanning Electron Microscopy. Results in Plate 1 show the comparison between Miscanthus fibres before and after this hydrolysis treatment.

Comparing SEM pictures of Miscanthus fibres to the insoluble fibres after hydrolysis it is clear the structure of the fibres has been preserved and the majority of the lignin has been removed although there appears to be some lignin globules that remains attached to the fibres as indicated in previous studies where (Micic et al., 2003; Xu et al., 2007). Each globule structure has a size between 0.6 μm

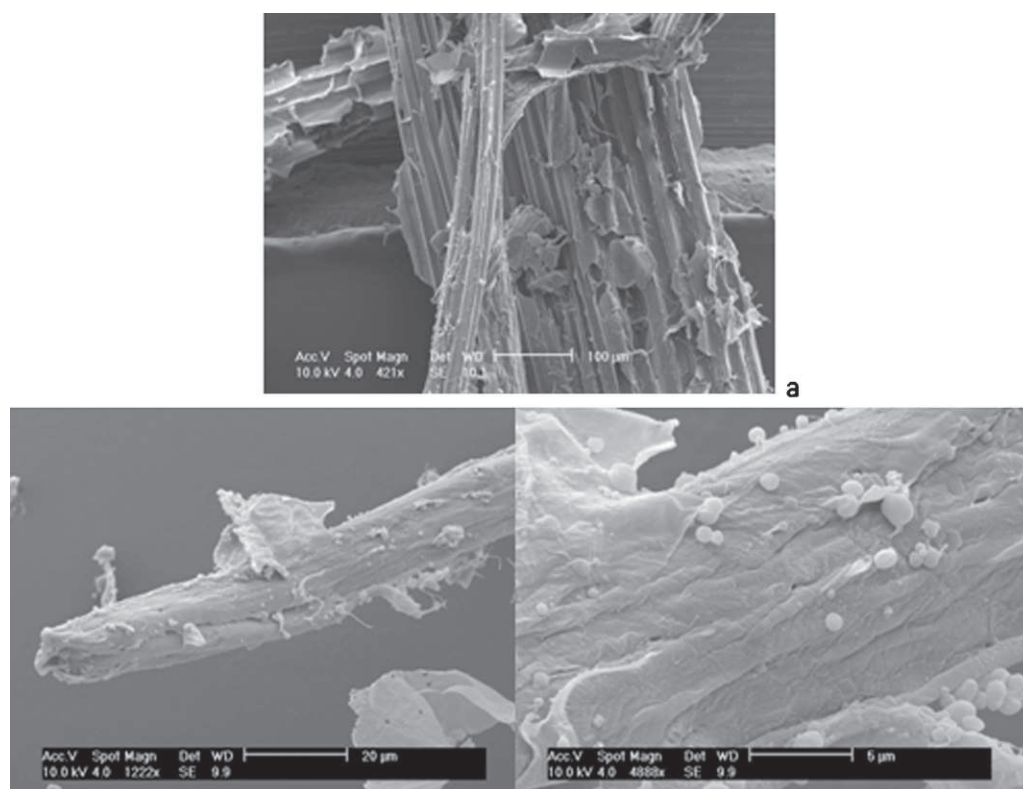


Plate 1. SEM pictures from (a) Miscanthus fibres before hydrolysis, (b) insoluble fraction fibres obtained after hydrolysis at 200 °C and biomass load of 15 g. Pictures were obtained by Philips XL-30 FEG ESEM microscope, operating at 10 kV acceleration voltage after coating by a gold sputter coater, Polaron SC 7640.

and 1 μm . Consequently, it falls in the range for the molecular lignin structures referred by Micic et al. (2003) of 0.02–1000 nm.

Therefore, the use of Sub-CW:ethanol:CO₂ mediated hydrolysis under the operating parameters described supports biomass delignification without destroying the cellulose fibres.

4. Discussion

Experiments show that temperature has a greater effect on biomass solubility than the biomass:water/ethanol ratio. Results indicate increasing temperature from 180 to 200 °C for a fixed biomass:solvent ratio, stimulated biomass solubilisation by 13% this observation correlates with the work of Pasquini et al. (2005). The work of Li and Kiran (1988) has shown a maximum solubilisation for red spruce wood of 19.3% at 190 °C and 290 bar, the Miscanthus solubility in our research varies between 35 and 53%.

Biomass delignification was also determined by comparing the amount of Klason lignin in biomass to the residual Klason lignin in the insoluble fraction. Results also indicate increasing temperature from 180 to 200 °C for a fixed biomass:solvent ratio, enhanced biomass delignification by 18%, obtaining a maximum delignification of 86% at 200 °C with load of 2.5 g per 250 ml of water/ethanol mixture was obtained. The results are in accordance with those reported by Pasquini et al. (2005) where a delignification extent of 93.1% for *P. taeda* wood chips and 88.4% for sugar cane bagasse. However, with Miscanthus the extent of delignification was obtained at lower pressures.

Finally, the insoluble fraction was also qualitatively analysed by SEM where globule/sphere structures were found. According to previous studies, these structures are believed to be former hydrolysed lignin that has been precipitated over the fibres afterwards. In addition, each globule structure has a size between 0.6 μm and 1 μm . Consequently, it falls in the range for the molecular lignin structures referred by Micic et al. (2003) of 0.02–1000 nm.

Consequently, the use of Sub-CW:ethanol:CO₂ mediated hydrolysis at these range of conditions is able to drive biomass delignification without destroying the cellulose fibres.

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